Remarks

The Amendments

Claim 41 has been amended to recite "Fas ligand" in place of a product which is detrimental to activated T cell proliferation in the patient." The amendment is supported by claim 43, which has been cancelled as redundant. Claim 41 has also been amended to recite that "a polynucleotide expressing Fas" is transferred into APCs. This amendment is supported by claim 44, which has been cancelled as redundant.

Claims 46, 48, 51, and 53 have been amended to perfect antecedent basis to amended claim 41.

Claim 53 has been amended to be dependent on claim 51 rather than now cancelled claim 52.

Claim 55 has been rewritten as an independent claim to include the elements of claim 54, now cancelled. Claim 66 has been amended to be dependent on claim 55 rather than now cancelled claim 54.

Claim 63 has been rewritten as an independent claim to include the elements of claims 58 and 60-62, which have been cancelled. Claims 59, 64, and 67 have been amended to be dependent on claim 63 rather than now cancelled claim 58.

None of these amendments introduce new matter.

The Rejection of Claims 42, 54-57, 60-63, and 66 Under 35 U.S.C. § 112, First Paragraph (Written Description)

Claims 42, 54-57, 60-63, and 66 are rejected under 35 U.S.C. § 112 as not adequately

described. Claims 54, and 60-62 have been canceled. Thus the rejection of these claims is rendered moot. Applicant respectfully traverses the rejection of claims 42, 55-57, 63 and 66.

Claim 42 is directed to a method of ablating auto-antigen-specific T cell in an auto-immune disease patient. Claims 55-57 are directed to antigen presenting cells. Claims 63 and 66 are directed to viruses. Each claim recites either APCs or viruses that comprise a polynucleotide which encodes "all or a portion of an auto-antigen" (referred to hereafter collectively as "auto-antigen"), a signal sequence and a transmembrane/cytoplasmic tail. The auto-antigen is functionally located with respect to the signal peptide and transmembrane/cytoplasmic tail such that the auto-antigen is processed by endosomes.

To comply with the written description requirement, the description must clearly convey to persons of ordinary skill in the art that applicants invented what is claimed. *In re Gosteli*, 872 F.2d 1008, 1012 (Fed. Cir. 1989). The description need only describe in detail that which is new or not conventional. *Hybritech v. Monoclonal Antibodies*, 802 F.2d 1834 (Fed. Cir. 1986); *Fonar Corp. v. General Electric Co.*, 107 F.3d 1949 (Fed. Cir. 1997).

The Office Action asserts that claims 42, 55-57, 63 and 66 are not adequately described because the specification fails to describe all possible structures that functionally locate the auto-antigen with respect to the signal peptide and transmembrane/cytoplasmic tail so that endosomal processing occurs.

> The specification fail[s] to provide an adequate description to teach the structures, the identifying characteristics for the claim language, considering all possible *structures* that may exist using modern biochemical and molecular biological techniques to functionally connect the two elements, one skill in the art could not readily envision what structures the claim[s] embrace or exclude, accordingly, the specification does not provide a reasonable guide for those seeking to practice the invention.

Paper 25, page 4, lines 6-14, emphasis in original.

Each of the rejected claims recites a polynucleotide encoding a polypeptide comprising an auto-antigen functionally located with respect to a signal sequence, and transmembrane/cytoplasmic tail. Thus the structures that link the auto-antigen to the signal sequence and transmembrane/cytoplasmic tail must be encoded by the polynucleotide. Polynucleotides only encode amino acids which are linked by peptide bonds. Thus the functionally linking structures must only include an amino acid linker or a direct peptide bond. One of skill in the art would have understood that applicants had possession of this genus of structures. These structures were well known at the time the application was filed.

Exhibits A-F were published before the December 3, 1997 effective filing date of the application. Each exhibit teaches a polynucleotide encoding a polypeptide comprising a peptide of interest functionally linked to a transmembrane/cytoplasmic tail. The functional linkage is demonstrated by the localization of each protein of interest to the lysosomes or endosomes.

Ruff et al. (*J. Biol. Chem.* (1997) *272*, 8671-8678; Exhibit A) teaches a polynucleotide encoding a fusion protein comprising the human immunodeficiency virus envelope glyprotein 160 (HIV gp160) linked to the LAMP transmembrane and cytoplasmic domains. Ruff teaches a polynucleotide for "expressing HIV gp160 as a chimeric protein containing the LAMP lysosomal targeting sequence." (Page 8675, column 1, lines 35.) Ruff also teaches that the LAMP-1 domains function to localize HIV-1 gp160 to lysosomes. "Cells that expressed the wild type gp160 (*panel B*) had the expected reticular staining pattern. In contrast, cells that expressed the gp160/LAMP chimera (*panel A)* had a vesicular staining pattern characteristic of the lysosomal localization of LAMP and LAMP chimeras." Page 8673, column 2, lines 21-25 of the *Results*;

See also Figure 2.)

Wu et al. (*Proc. Natl. Acad. Sci. USA* (1995) *92*, 11671-11675; Exhibit B) teaches a polynucleotide encoding a chimeric polypeptide containing an antigen, human papillomavirus 16 E7 (HPV-16 E7), and the transmembrane/cytoplasmic domains of LAMP-1. "We therefore engineered a chimeric gene encoding a model antigen linked to the transmembrane and cytoplasmic region of LAMP-1." (Page 11671, column 2, lines 22-24.) Wu also teaches that linking HPV-16 E7 to the transmembrane/cytoplasmic domains of LAMP1 targets HPV-16 E7 to the endosomes and lysosomes of cells. "As expected, cells transfected with wt E7 showed homogeneous cytoplasmic/nuclear staining (Fig. 2*B*). In comparison, cells transfected with the chimeric Sig/E7/LAMP-1 construct displayed a vesicular pattern consistent with endosomal and lysosomal localization (Fig. 2*A*)." (Page 11672, column 2, lines 8-13.)

Conibear et al. (*Journal of Cell Science* (1994) *107*, 923-932; Exhibit C) teaches a polynucleotide encoding a lysozyme protein (lys) linked to the transmembrane/cytoplasmic tail of the mannose 6-phosphate/IGF-II receptor (MPR). The connection of MPR to the cytoplasmic and transmembrane domains of lys functionally locates MPR to the trans Golgi region of cells.

> We fused the cytoplasmic and transmembrane domains of the bovine mannose 6-phosphate/IGF-II receptor (MPR) to lysozyme, a monomeric secretory protein thought to be devoid of sorting information. When the resulting chimera (lys/MPR) was transiently expressed in COS cells or stably expressed in CV1 cells, it had a predominantly intracellular distribution in the trans-Golgi region.

Page 923, lines 1-7 of the Abstract.

Peters et al. (*EMBO J* (1990) *9*, 3497-3506; Exhibit D) teaches that a polynucleotide encoding a chimeric polypeptide comprising mannose 6-phosphate receptor (MPR46) linked to

the transmembrane and cytoplasmic domains of lysosomal acid phosphatase (LAP). "A chimeric protein consisting of the ectoplasmic domain and 6 amino acids of the transmembrane domain of MPR46 and the complete transmembrane and cytoplasmic domains of LAP was constructed." (Page 2501, column 2, lines 17-21.) Peters also teaches that linking MPR46 to the transmembrane and cytoplasmic domains of LAP functions to target MPR46 to the lysosomes. "These observations show that the MPR46-LAP chimers are delivered to lysosomes, while the wild type MPR46, as well as receptors lacking a cytoplasmic domain, are excluded from lysosomes." (Page 3503, column 1, lines 1-6.)

Hatem et al. (*Journal of Cell Science* (1995) *108*, 2093-2100; Exhibit E) teaches the construction of a polynucleotide to produce a protein in transfected cells containing chicken LEP100 linked to the transmembrane and cytoplasmic domains of LAMP-2. "The chimeric proteins contain the lumenal domain of chicken LEP100, . . . The transmembrane domain and the cytoplasmic domain were derived from the LAMP-2 molecules." (Page 2096, column 1, line 7 to column 2, line 4.) Expression of the polynucleotide demonstrates that the transmembrane and cytoplasmic domains of LAMP-2 function to target LEP100 to the lysosomes. "When the transfected cells were labeled with LEP100-mAB, a pattern of fluorescent labeling typical of lysosomes was observed. . . . Thus, the transmembrane and cytoplasmic domains of each of the LAMP-2 variants are sufficient to target chimeric proteins to lysosomes." (Page 2098, column 1, lines 4-13.)

Vijayasaradhi *et al.* (*J. Cell. Biol.* (1995) *130*, 807-820; Exhibit F) teaches a polynucleotide encoding a fusion protein that contains the extracellular domain of CD8 and the transmembrane and cytoplasmic domains of gp75. "[W]e constructed chimeric proteins

consisting of the extracellular domain of the T lymphocyte surface glycoprotein CD8, transmembrane (TM) domains CD8 or gp75, and cytoplasmic tail (Cyt) of gp75." (Page 808, column 1, lines 21-26.) Vijayasaradhi teaches that CD8 is functionally linkd to the gp75 transmembrane/cytoplasmic domains because CD8 is localized to the endosomal/lysosomal compartments when fused to the gp75 domains. "In fibroblast transfectants, chimeric CD8 molecules containing the 36-amino acid cytoplasmic domain of gp75 were retained in cytoplasmic organelles. Signals in the gp75 cytoplasmic tail alone, were sufficient for intracellular retention and targeting of the chimeric proteins to the endosomal/lysosomal compartment." (Page 807, lines 18-23.)

Alberts et al. (Molecular Biology of the Cell (1994) 3rd ed., Figure 2-7; Exhibit G) also teaches that peptide bonds were known in the art at the time the application was filed and thus one of skill in the art would have understood that the Applicant also had possession of this structure that functionally links or connects polypeptides. Alberts et al. teaches the structure of a peptide bond connecting two amino acid residues.

The genus of structures encoded by a polynucleotide that functionally locates an auto-antigen to a signal sequence and transmembrane/cytoplasmic tail was known in the art at the time the application was filed. Thus one of skill in the art would understand that the applicant was in possession of the recited genus of structures in claims 42, 55-57, 63 and 66.

Withdrawal of this rejection is respectfully requested.

The Rejection of Claims 42, 54-57, 60-63, and 66 Under 35 U.S.C. § 112, First Paragraph (Enablement)

Claims 42, 54-57, 60-63, and 66 are rejected under 35 U.S.C. § 112 as not enabled. Claims 54, and 60-62 have been canceled. Thus the rejection of these claims is rendered moot. Applicant respectfully traverses the rejection of claims 42, 55-57, 63 and 66.

Claim 42 is directed to a method of ablating auto-antigen-specific T cell in an autoimmune disease patient. Claims 55-57 are directed to antigen presenting cells. Claims 63 and 66 are directed to viruses. Each claim recites either APCs or viruses that comprise a polynucleotide which encodes "all or a portion of an auto-antigen" (referred to hereafter collectively as "auto-antigen"), a signal sequence and a transmembrane/cytoplasmic tail. The auto-antigen is functionally located with respect to the signal peptide and transmembrane/cytoplasmic tail, such that the auto-antigen is processed by endosomes.

To satisfy the enablement requirement, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. *In re Wright*, 999 F.2d 1557 (Fed. Cir. 1993). A specification need not disclose what is well known to those skilled in the art and preferably omits that which is well known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986).

The Office Action asserts that the claims are not enabled because the specification does not teach a sufficient number of structures that can be used to functionally locate the signal sequence or transmembrane/cytoplasmic tail to the auto-antigen. The Office Action alleges that

"except the illustrated embodiment, one would not know how to use the invention without first carrying out undue experimentation to determine which of the structures would have the recited function." (Paper 25, page 5, lines 18-21.)

As discussed above, the genus of structures recited by the rejected claims encompasses only structures that can be encoded by a polynucleotide because as recited in the claims the auto-antigen functionally linked to the signal peptide and transmembrane/cytoplasmic domain is encoded by a polynucleotide. Thus these structures include (1) peptide bonds and (2) amino acid linkers. Exhibits A-F, discussed above with respect to the written description requirement, demonstrate that these structures were well known and used by those in the art to localize proteins at the time the application was filed. Thus it would not have required undue experimentation for one of skill in the art to determine what structures have the recited function.

Furthermore, one of skill in the art would have been able to make and use polynucleotides encoding polypeptides having these structures. Exhibits A-F provide evidence that at the time the application was filed it was routine to make and use polynucleotides encoding polypeptides in which a peptide of interest is functionally linked to a transmembrane/cytoplasmic domain.

Ruff et al. (Exhibit A) teaches a polynucleotide encoding HIV-1 gp160 functionally linked to the LAMP-1 transmembrane/cytoplasmic domains. "The gp160/LAMP chimeric gene construct was prepared by ligating the sequence encoding the entire extracellular domain of HIV-1 LAI gp160 to the sequence encoding the transmembrane/cytoplasmic domains of the murine lysosomal membrane glycoprotein, LAMP-1 as described in the methods." (Page 8673, column 2, lines 1-6 of the Results; See also Figure 1 and Page 8672, column 1, line 9 through

column 2, line 7 of the EXPERIMENTAL PROCEDURES.)

Wu et al (Exhibit B) teaches a polynucleotide encoding HPV-16 E7 functionally linked to the signal peptide and transmembrane/cytoplasmic tail of LAMP-1.

> For generation of the Sig/E7/LAMP-1 chimeric gene, DNA fragments encoding the signal peptide (N-terminal) of LAMP-1, the open reading frame of HPV-16 E7, and the transmembrane domain and cytoplasmic tail of LAMP-1 were amplified by PCR using high-fidelity *Pfu* polymerase (Stratagene). The primer sets were designed so that *Bgl*II and *Bam*HI restriction sites were generated at the 5' and 3' ends of the amplified fragments, respectively. The primer set for signal peptide of LAMP-1 was 5'-TTGAGATCTTATGGCGGCCCC-3' and 5'-TTGGGATCCTCAAAGAGTGCTGA-3'. The primer set for HPV-16 E7 open reading frame was 5'-CCCAGATCTAATCATGCATG-3' and 5'-TATGGATCCTGAGAACAGAT-3'. The primer set for the transmembrane and cytoplasmic domain of LAMP-1 was 5'-TCAAGATCTTAACAACATGTTG-3' and 5'-TGTGGATCCCTTCCACACC-3'. The amplified DNA fragments were cloned sequentially into the unique *Bam*HI cloning site of the pCMVneo expression vector downstream of the cytomegalovirus promoter.

Page 11671, column 2, line 1 to page 11672, column 1, line 9 of the MATERIALS AND METHODS; See also Page 11672, column 2, lines 9-13 of the RESULTS and Figure 1.)

Conibear et al. (Exhibit C) teaches a polynucleotide encoding a lysosomal protein functionally linked to the transmembrane/cytoplasmic tail of MPR. "A chimeric 'lys/MPR' molecule was created by fusing the transmembrane and cytoplasmic domains of the bovine MPR to the C terminus of chicken lysozyme, leaving 4 residues of the MPR lumenal domain to provide a spacer between lysozyme and the membrane." (Page 925, column 2, lines 4-8 of the Results.) Conibear also provides a detailed method that teaches how to make a protein having the recited linking or connecting structures.

> Fusion of lysozyme and receptor sequences took advantage of the termination codon of the lysozyme cDNA in PSAYI forming part of the *Xba*I site. By cutting with *Xba*I and blunting with mung bean nuclease, the stop codon as well as one other base at the 3' end of the coding sequence is removed. *Bam*HI sites at position 2337 in the LDLR sequence and at position 3811 in the MPR sequence were created by site-directed mutagenesis in M13mp18 by the method of Zoller and Smith (1984). When these *Bam*HI sites were cut and blunted with mung bean nuclease, an extra base was left at their 5' ends. This allowed fusion of the lysozyme sequence with each receptor sequence, restoring the last residue of the lysozyme sequence and maintaining the reading frame. Thus fragments carrying receptor sequences cut with *Bam*HI, treated with mung bean nucleotide and cut with *Eco*RI were ligated into SAYI that had been cut with *Xba*I, blunted, and cut with *Eco*RI. The resulting vectors, p1/M and p1/L, are able to direct the expression of lys/MPR and lys/LDLR chimeras in COS cells.

Page 924, column 1, lines 21-37.

Peters (Exhibit D) teaches a polynucleotide encoding the ectoplasmic domain of the mannose 6'phosphate receptor functionally linked to the transmembrane/cytoplasmic domain of LAP. Peters teaches:

> For construction of the MPR46-LAP chimera a PstI site was introduced at position 587 of the MPR46 cDNA. The MPR46 cDNA encoding the N-terminal part of the receptor was fused at this artificial PstI site to the unique PstI site for the LAP cDNA fragment encoding the C-terminal part of the protein. The construct encodes for the N-terminal 197 amino acids of MPR46 (with exchanges of phenylalanine 196 to serine), followed by the transmembrane and cytoplasmic domain of LAP (see Figure 2).

Page 3505, column 2, lines 21-28.

Hatem (Exhibit E) teaches a polynucleotide encoding the lumenal domain of LEP100 functionally linked to the transmembrane/cytoplasmic domain of LAMP-2. Hatem teaches:

> The chimeras were generated by splicing the chicken LAMP-1 cDNA (LEP100) encoding the lumenal domain to the portion of the chicken LAMP-2 cDNA encoding the transmembrane domain,

> cytoplasmic domain and 3' untranslated region. Restriction sites for the enzyme *Hpa*I were introduced into the LEP100 and LAMP-2 cDNAs by PCR. This resulted in changing Gly363 to Val and Asp364 to Asn in LEP100 and Glu373 to Val in the LAMP-2 proteins (numbering with the start methionine as the first amino acid). The chimeras contained only the Gly363 to Val substitution.

Page 2094, column 2, lines 26-34.

Vijayasaradhi (Exhibit F) teaches a polynucleotide encoding the amino-terminal extracellular domain of CD8 functionally linked to the transmembrane and cytoplasmic domains of gp75. Vijayasaradhi teaches:

> Chimeric cDNAs encoding fusion proteins CD8/gp75 (TM + Cyt), and CD8/gp75 (Cyt) were constructed by the following methods. First, appropriate restriction sites at or near the TM/Cyt junction of CD8, and luminal/TM and TM/Cyt junctions of gp75 were generated by site-directed mutagenesis using Mutagene kit. . . . Plasmid pSVgp75RV was digested with EcoRV and XbaI to produce an ~1.2-kb fragment containing the TM + Cyt sequence and 3' untranslated sequence of gp75 cDNA, including part of the multiple cloning site sequences of the vector; plasmid pSVleu2H was digested with EcoRV and XbaI and the large ~4-kb plasmid DNA fragment lacking TM and Cyt sequences of CD8 cDNA was isolated. The 1.2-kb EcoRV-XbaI gp75 fragment was ligated with the large EcoRV-XbaI pSVleu2H fragment to generate a plasmid construct encoding the fusion protein CD8/gp75 (TM + Cyt).

Page 808, column 2 lines 17-51, citations omitted.

Thus it was well known, at the time the application was filed, how to make and use polynucleotides encoding polypeptides having structures that functionally locate a peptide of interest (*e.g.*, auto-antigen) with respect to a signal sequence and/or a transmembrane/cytoplasmic tail. It would not have required undue experimentation for one of skill in the art to make and use these polynucleotides or the polypeptides encoded by them.

Withdrawal of this rejection of claims 42, 54-57, 60-63, and 66 is respectfully requested.

The Rejection of Claims 41-53 and 65 Under 35 U.S.C. § 112, First Paragraph (Enablement)

Claims 41-53 and 65 stand rejected under 35 U.S.C. § 112, first paragraph as not enabled by the specification. Claims 43-45, 47, and 52 have been cancelled. Thus the rejection of these claims is rendered moot. Applicant respectfully traverses the rejection of claims 41, 42, 46, 48-51, 53, and 65.

Claim 41 is the only independent claim of the rejected claim set. Claim 41 is directed to a method of ablating auto-antigen-specific T cells in an autoimmune disease patient. Antigen presenting cells (APCs) are removed from an autoimmune disease patient. Polynucleotides are encoding all or a portion of an auto-antigen and Fas ligand are transferred into the APCs. The APCs are reintroduced into the patient. The patient's auto-antigen-specific T cells are activated and ablated.

To satisfy the enablement requirement an inventor must provide sufficient information about the claimed invention that a person of skill in the field of the invention can make and use it without undue experimentation, relying on the patent specification and the knowledge in the art. *Scripps Clinic & Research Foundation v. Genentech, Inc.* 927 F.2d 1565 (Fed. Cir. 1991). A specification need not disclose what is well known to those skilled in the art and preferably omits that which is well known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367 (Fed. Cir. 1986). To establish a *prima facie* case of non-enablement, the Patent Office has the initial burden to establish a reasonable basis to question the enablement provided in the specification. *In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993).

The Office Action asserts that the method claims are not enabled because the claims recite broad methods conducted in an auto-immune disease patient but the specification "does not reasonable provide enablement for ablation of autoreactive T cell in *any* and *all* auto-immune disease patients using *any* and *all* antigen-presenting cells, *any* and *all* viral vectors, *any* and *all* signal sequences, *any* and *all* auto-antigens; and it does not reasonably provide enablement for selective ablation of auto-antigen-specific T cells in an auto-immune disease patient using *any* and *all* T cell detrimental products." (Paper 25, page 6, lines 8-13, emphasis in original.) The Office Action points to twelve separate reasons why the claims are not enabled. Each will be discussed in turn.

(1) The Office Action asserts that the claims are not enabled because the declaration executed January 3, 2002 (Exhibit H) is deficient. The declaration provides evidence that administration of APCs expressing HA, Fas ligand, and truncated FADD to transgenic mice that contain T cells expressing a receptor for HA causes the T cells expressing a receptor for HA to be ablated. First, the Office Action asserts that the declaration does not enable the claims because the declaration does not teach whether the "HA-receptor bearing T cells are representative of HA activated T cells." (Paper 25, page 9, lines 10-11.) The Patent Office, however, has presented no evidence or sound scientific reasoning to doubt that the HA-receptor bearing T cells are activated. HA receptor bearing T cells must be activated because they are ablated in the mice and only activated T cells are susceptible to ablation by Fas ligand.

Second, the Office Action asserts that the declaration is not sufficient to enable the claims because it is not clear if the HA-specific T cells can be ablated over a time "period beyond 8 days." (Paper 25, page 9, lines 12-13.) Applicant respectfully submits that the method merely

requires ablation of auto-antigen-specific T cells in an autoimmune disease patient. The method does not require ablation of auto-antigen-specific T cells over a particular period of time following administration, *e.g.*, beyond eight days. The declaration provides evidence that an antigen-specific T cell is ablated 2, 5, and 8 days after administration of an APC expressing the antigen and Fas ligand. Thus the declaration provides evidence that antigen-specific T cells can be ablated in an animal, as is claimed in the methods.

Third, the Office Action asserts that the HA transgenic mouse study described in the declaration cannot enable the claims because the claims' scope is too broad. Applicant maintains that the declaration does support the broad method claims because it demonstrates a simple principle that can be applied to any antigen, including auto-antigens. APCs present antigens to T cells. APCs that present antigen "X" engage T cells that have a receptor for antigen "X". These APCs then stimulate these T cells. If the APCs also express Fas ligand, they will ablate the T cells. The specification of the application provides evidence that this scenario is true in cell culture systems. The declaration provides evidence that this scenario is true in an animal. The Patent Office has provided no reason to doubt that this will also occur in autoimmune disease patients.

(2) The Office Action asserts that the claimed methods are not enabled because one cannot predict whether polynucleotides can be transferred to APCs as recited in the method claims and if the APCs will be targeted to auto-antigen specific T cells in vivo. (Paper 25, page 9, line 22 to page 10, line 4.) First, it is entirely predictable that polynucleotides will be delivered to the APCs. The Applicant has demonstrated *ex vivo* transfer of polynucleotides to APCs at page 12, line 17 through page 13, line 4 of the specification. Applicant has also demonstrated transfer of

polynucleotides to APCs in the declaration (Exhibit H). Furthermore, Exhibits I-K demonstrate that before the effective filing date of the application, December 3, 1997, it was known in the art how to transduce or transfect APCs.

Reeves et al. (*Cancer Res.* (1996) *56*, 5672-5677; Exhibit I) teaches retroviral transduction of human dendritic cells to transfer a polynucleotide encoding a melanoma tumor associated antigen (TAA). Reeves teaches that "human DCs [dendritic cells] can be retrovirally transduced with a TAA gene and that these transduced cells can raise a specific antitumor response in vitro." (Lines 21-23 of the Abstract.)

Wan et al. (*Hum. Gene Ther.* (1997) *8*, 1355-1363; Exhibit J) teaches transduction of human dendritic cells with an adenoviral vector. Wan teaches "We have used replication-defective adenovirus vectors (Ads) to transduce DCs with various genes, including tumor antigen genes. We found that 90% of murine bone marrow derived-DCs could be infected with an Ad vector." (Lines 6-9 of the Abstract.) Wan further teaches that the "modified DCs appear nontoxic and stimulate a potent antitumor response." (Lines 23-24.)

Bello-Fernandez et al. (*Hum. Gene Ther.* (1997) 8, 1651-1658; Exhibit K) teaches transduction of dendritic cells with a retroviral vector. "A retroviral-vector encoding the low affinity nerve growth factor receptor (LNGFR) was used to transduce dendritic cells (DCs) generated from CD34+ cord blood (CB) progenitor cells under serum-free conditions." (Lines 1-3 of the abstract.) Bello-Fernandez further teaches that "transduced CD1a+ DCs maintained their functional properties, stimulating allogeneic T cells with similar efficiency as nontransduced CD1a+ DCs. Thus, the serum-free system described allows efficient generation and transduction of CD1a+ DCs derived from CD34+ progenitor cells." (Lines 20-24.)

Thus it was well known in the art that APCs could be isolated and transduced or transfected with a vector. There is no reason to doubt that the APCs recited in the claims can also be transduced or transfected with a polynucleotide.

The Office Action has also expressed doubt that the APCs transfected with the auto-antigen will be targeted to auto-antigen-specific T cells in an autoimmune disease patient. The Office Action asserts that "the T cells of the mouse used in the study bear specific HA-receptors, which receptors promoted targeting of administered APCs to these cells, thus facilitated the FasL cell-killing effect. . . . in an auto-immune patient, such targeting mechanism is absent, therefore, the effect of intraperitoneal or intravenously injection of transduced APCs in the patient is unpredictable." (Paper 25, page 9, line 19 to page 10, line 2.) Contrary to the statements in the Office Action, the mechanism which promoted targeting of the APCs administered to the mice in the HA transgenic mouse study is identical to mechanism which promotes targeting of the APCs to auto-antigen-specific T cells in an autoimmune disease patient. In each instance the transduced or transfected APCs express an antigen (HA or auto-antigen) that is recognized by T cells having a specific receptor (for HA or the auto-antigen). In each instance the T cells expressing the receptor for the specific antigen (HA or auto-antigen) engage the APC that presents the specific antigen (HA or autoantigen). This recognition and engagement results in activation and subsequent ablation of the specific T cells. The Patent Office provides no reason to doubt that this targeting mechanism will work similarly in an autoimmune disease patient as in the mouse model.

(3) The Office Action asserts that the claimed methods are also not enabled by the *in vivo* HA transgenic mouse study (Exhibit H) because the declaration does not teach *how* the HA-

specific $CD4^+$ T cells are ablated in the HA transgenic mouse. The Office Action specifically asserts that because the mice treated with APCs expressing FasL and FADD (and not HA) show a decrease in the percentage of HA-specific $CD4^+$ T cells over eight days it is possible that the mechanism by which the mice treated with APCs transduced with a polynucleotide encoding HA, FasL and truncated FADD have decreased HA-specific $CD4^+$ cells is nonspecific. First, the mice in the treatment group have approximately half as many HA-specific $CD4^+$ T cells as each group of control mice at all times in the study. Second, the declaration, at Table 2, shows that proliferation of HA-specific splenocytes and lymph node cells was significantly decreased in the mice treated with APCs expressing HA, FasL, and truncated FADD compared to mice treated with APC expressing FasL and truncated FADD or mice not treated with APCs. These data supplement Table 1 and support the hypothesis that HA-specific $CD4^+$ cells are specifically targeted and ablated.

(4) The Office Action also asserts that the HA transgenic mouse study is insufficient to enable the claimed methods because "the preamble of claim 1 calls for removal of auto-antigen-specific T cells, although on day 2 after transduced APCs administration, HA-receptor bearing T cells are significantly decreased to 20% of original, they recovered to 50% of the original on day 8." (Paper 25, page 11, lines 18-21.) Because the mouse treated with APCs expressing HA-LAMP-sig, FasL, and truncated FADD had approximately 20% of the HA specific $CD4^+$ T cells of either of the control mice, 80% of its HA specific $CD4^+$ T cells were removed, *i.e.* ablated, relative to either of the control mice. This is the ablation of T cells recited in the claimed methods. The methods do not require that a certain percentage of auto-antigen-specific T cells be ablated or that the auto-antigen-specific T cells be ablated for a particular period of time.

Thus, Table 1 shows the ablation of HA-specific T cells in the HA transgenic mice as recited in the claims.

(5) The Office Action asserts that the claims are not enabled for using any APCs in the claimed methods. Methods of isolating APCs were well known in the art at the time the application was filed and thus need not be disclosed in the specification. The Patent Office has reiterated its position that the specification is required to teach how to isolate various types of APCs without any evidence or sound scientific reasoning to demonstrate that these methods were not known.

Exhibits L-P were all published before the December 3, 1997 effective filing date of the application. Each exhibit demonstrates that methods of isolating different types of APCs (e.g., Langerhans cells, keratinocytes, skin dendritic cells, and B cells) were known in the art at the time the application was filed.

Simon et al. (*Exp. Dermatol.* (1995) *4*, 155-161; Exhibit L) teaches a method of purifying Langerhans cells (LC) from human skin. Simon teaches, "To isolate fresh LC (fLC), epidermal cell suspensions (EC) were stained with anti-C1a mAb and with appropriate secondary reagents conjugated to microbeads and to FITC. They were then passed over a separation column and exposed to a strong magnetic field. Thereafter both CD1a-depleted and CD1a-enriched cells were collected." (Lines 9-13 of the Abstract.) Simon concludes that "MACS [magnetic cell sorting]-separation is an efficient and rapid method to isolate human fLC and cLC [cultured Langerhans cells] of high purity and unimpaired function." (Lines 24-26.)

Hanau et al. (*J. Invest. Dermatol.* (1988) *91*, 274-279; Exhibit M) also teaches a method purifying Langerhans cells from human epidermis. Hanau teaches, "Because Langerhans and

indeterminate cells are the only epidermal cells that express the specific CD1a surface antigen T6, we have used immunomagnetic monodisperse polymer microspheres for positive selection of human epidermal Langerhans and indeterminate cells. Epidermal cells in suspension are successively incubated with a murine monoclonal anti-T6 antibody of the IgG1 subclass and then with magnetic beads coated with a sheep anti-mouse IgG1. Rosetted cells are obtained and then easily separated from the non-rosetted cells using a magnet. . . . All the rosetted cells (1.5 to 5% of the total epidermal cells) express T6 antigen by indirect immunofluorescence and under the electron microscope possess all the ultrastructural characteristics of Langerhans cells. Moreover, the rosetted Langerhans cells remain functional." (Lines 1-14 of the Abstract.)

Normand et al. (*In Vitro Cell Dev. Biol. Anim.* (1995) *31*, 447-455; Exhibit N) isolated keratinocytes from human skim. Normand teaches that "[t]o isolate and propagate keratinocytes from a punch biopsy, the epidermis was separated from the dermis by treatment with dispase. Keratinocytes were dissociated from the epidermis by trypsin and plated on a collagen-coated tissue culture petri dish. A combination of two commercial media (Serum-Free Medium and Medium 154) provided optimal growth conditions." (Lines 6-11 of the Abstract.)

Karasek (*J. Invest. Dermatol.* (1983) *81*, 24s-8s; Exhibit O) teaches that different methods of isolating keratinocytes were known in the art prior to and including 1983. Karasek teaches, "Human keratinocytes and those from several laboratory animal species may now be isolated from skin either by direct trypsinization of minces, from split-thickness skin following the separation of the epidermis from the dermis, or from the outgrowth form tissue explanted in liquid medium." (Lines 2-6 of the Abstract.)

Richters et al. (*Clin. Exp. Immunol.* (1994) 98, 330-336; Exhibit P) teaches a method to

isolate human skill dendritic cells. Richters teaches, "A method is described to isolate and characterize human skin dendritic cells (DC). This method is based on the migratory capacities of these cells. The cells migrated 'spontaneously' out of the split-skin explants into the medium during a 24-h culture period and contained up to 75% CD1a+ cells. After removal of co-migrated T cells and macrophages, the highly enriched (> 95% CD1a+) DC showed potent allo-antigen-presenting capacities." (Lines 1-6 of the Abstract.)

Schultze et al. (J. Clin. Invest. (1997) 100, 2757-2765; Exhibit Q) teaches the isolation of human B cells from peripheral blood mononuclear cells (PBMCs).

> Before adding PBMC, t-CD40L cells [NIH3T3 cells transfected by electroporation with the human CD40 ligand] were washed twice by rinsing the plates with PBS. CD40-Bs [CD40 activated B cells] were generated from PBMC by simply coculturing whole PBMC at 2x 10^6 cells/ml with t-CD40L in the presence of IL-4 (2 ng/ml; Immunex, Seattle, WA) and cyclosporin A (CsA) at 5.5 x 10^{-7} M in Iscove's MDM (Gibco/BRL) supplemented with 10% human AB serum, 50 µg/ml transferring (Boehringer Mannheim, Indianapolis, IN), 5 µg/ml insulin (Sigma Chemical Co., St. Louis, MO), and 15 µg/ml gentamicin (Gibco/BRL) at 37°C in 5% CO_2. . . . Cultured cells were transferred to new plates with fresh irradiated t-CD40L cells every 3-5 d.

Page 2758, column 1, lines 18-31.

Thus methods of isolating APCs were well known in the art as of the effective filing date of the application. Thus the claims are enabled for using any APCs to perform the claimed methods.

(6) The Office Action also asserts that the claims are not enabled for using any auto-antigen and for treating any type of autoimmune disease.

The Office Action alleges that the claims are not enabled for any type of autoimmune

disease because "[t]he mechanisms leading to these diseases vary and cover almost every step of cellular and humoral immune responses, from various auto-antibodies to complements, to subtypes of T cells." (Paper 25, page 12, lines 8-11.)

The claimed methods are directed to "ablating auto-antigen-specific T cells in an auto-immune disease patient." Thus the claimed methods are directed to treating an autoimmune disease patient having auto-antigen-specific T cells. It may be true that the mechanisms that lead to different autoimmune diseases are different. However, this fact has no bearing on whether, if an autoimmune disease patient has auto-antigen-specific T cells, the auto-antigen-specific T cells will be ablated by an antigen presenting cell expressing the auto-antigen and Fas ligand.

The Office Action also alleges that not every auto-antigen known in the art could be used in the suppression of an unwanted immune response. The Office Action cites Von Herrath (Ann. Med. (2000) 32, 285-292) to support its assertion. Von Herrath is cited as teaching that not all auto-antigens can be used to suppress an unwanted autoimmune response. (Paper 25, page 12, lines 18-23.)

Von Herrath teaches that different auto-antigens are involved in either a regulatory autoimmune process or an autoaggressive immune process in an autoimmune disease model for diabetes. For example, Von Herrath teaches that "DNA vaccines with plasmids expressing the insulin B-chain but not the LCMV NP self-antigen can prevent IDDM in rat insulin promoter (RIP) LCMV transgenic mice. Responses to the LCMV NP antigen, which is the primary triggering islet (self)-antigen in the RIP LCMV mice, are invariably of the Th1/Tc1 autoaggressive type." (Page 288, column 1, line 30 to page 289, column 2, line 5.) Even if, *arguendo*, Von Herrath teaches that not all auto-antigens can be used in the claimed methods to

ablate auto-antigen-specific T cells, the Patent Office has provided no reason to support that one of skill in the art would have been unable to determine which auto-antigens to use in the claimed methods.

In fact, auto-antigens for which autoaggressive T cells are specific were known in the art before the December 3, 1997 effective filing date of the application. The specification teaches that "[t]he extracellular portion of the α-subunit (comprising amino acids 1-210) is believed to comprise the epitopes to which most AchR-specific T cells respond." (Page 6, lines 18-20.)

Adamizu et al. (*Thyroid* (1995) *5*, 259-264; Exhibit R) teaches that auto-antigen-specific T cells in Grave's disease recognize specific epitopes of the thyrotropin (TSH) receptor. T cells "reacted well against three groups [of epitopes]: the N-terminal (amino acids 31-169) and C-terminal (338-420) regions of the extracellular domain and the N-terminal half (441-661) of the transmembrane domain of the receptor." (Lines 8-11 of the Abstract.)

Merkel et al. (*Kidney Int.* (1996) *49*, 1127-1133; Exhibit S) teaches that auto-antigen-specific T cells in Goodpasture's syndrome autoimmune disease patients react with the N-terminal NC1 domain of alpha 3 type IV collagen. "This [T cell] clone specifically recognized a motif at the N-terminal area of the alpha 3 (IV) NC1 domain (AA 51 to 59: GSPATWTTR). We conclude that autoreactive T-cells exists in Goodpasture patients and may play a crucial role in the inflammatory process. T-cell clones are autoreactive to the alpha 3 (IV) NC1 domain." (Lines 13-17 of the Abstract.)

Wucherpfennig et al. (*Ann. N. Y. Acad. Sci.* (1995) *756*, 241-258; Exhibit T) teaches that an auto-antigen-specific T cell in multiple sclerosis recognizes myelin basic protein. Wucherpfennig teaches that "the T-cell response to MBP [myelin basic protein] was focused on

the MBP (84-102) peptide and in vivo expanded population(s) dominated the response to the MBP (84-102) peptide." (Lines 13-15.)

Thus auto-antigens to which auto-antigen specific T cells respond were known in the art at the time the application was filed. There is no reason to doubt that one of skill in the would have been able to select an auto-antigen with which to practice the claimed invention.

(7) The Office Action asserts that some of the rejected claims are not enabled because they broadly recite a signal sequence and it is *highly* unpredictable whether any signal sequence fused to any auto-antigen will result in presentation of the auto-antigen on an APC. (Paper 25, page 13, lines 7-9.) The Office Action cites Chaux (U.S. Patent 6,426,217) to support its assertion. Chaux is cited as teaching "fusion proteins of MAGE-3 and human invariant chain Ii, but not LAMP-1, are efficiently targeted to the HLA class II peptide presentation pathway." (Paper 25, page 13, lines 5-7.) It is true that Chaux teaches an example in which a signal sequence, LAMP-1, does not facilitate MHC presentation of an antigen on a cell. However, it is not true that Chaux teaches that it was *highly unpredictable* that any signal sequence can be fused to any antigen for MHC presentation in a cell. In fact, Chaux teaches that it was surprising that the MAGE-3-LAMP-1 fusion proteins were not displayed. "Surprisingly, fusion of an endosomal targeting portion of LAMP-1 protein did not significantly increase targeting of MAGE-3 to the HLA class II peptide presentation pathway." (Column 11, lines 25-27, emphasis added.) Thus, if anything, Chaux teaches that it was *predictable* that fusing a portion of LAMP-1 protein to a protein of interest would target the protein of interest for presentation and that MAGE-3 is an *exception* to what was known in the art. Chaux, therefore, teaches that it is not *100% predictable* that fusion of an auto-antigen to a signal sequence will result in presentation of the auto-antigen.

Chaux does not however teach that it is *highly unpredictable* that fusion of an auto-antigen to a signal sequence will result in presentation of the auto-antigen.

(8) The Office Action asserts that one of skill in the art would have to take multiple factors into account when determining a vector to transduce or transfect polynucleotides into an APC. The Office Action cites Makrides (*Protein Exp. Pur.* (1999) *17*, 183-202) and Robbins (*Pharmcol. Ther.* (1998) *80*, 35-47) as teaching factors to consider when choosing a viral vector to treat an autoimmune disease patient. (Paper 25, page 14, lines 1-2.) Applicant agrees that there are many factors to consider when determining a viral vector (*if* a *viral* vector is used) to deliver polynucleotides to an APC. One of skill in the art would have been able select such a viral vector using the teachings in the specification and the well-known characteristics of different viral vectors at the time the application was filed. The specification teaches that vaccinia virus can be used to transfer polynucleotides to APCs. Furthermore, Makrides and Robbins review known advantages and disadvantages of using different viral vectors to transfer genes to a cell. Having this knowledge, one of skill in the art would have been able to select an appropriate vector to transfer polynucleotides to an APC as recited in the claimed methods.

(9) The Office Action also asserts that the specification does not enable the recitation "a product which is detrimental to activated T cell proliferation in the patient." The method claims have been amended to recite "Fas ligand" in place of "a product which is detrimental to activated T cell proliferation in the patient."

(10) The Office Action also asserts that the specification does not enable the claims because it does not teach when to administer the APCs that express the autoantigen and Fas ligand to an auto-immune disease patient. The Office Actions cites two references, Von Herrath (*Ann. Med.*

(2000) *32*, 285-292) and Kristiansen (*Genes and Immunity* (2000) *1*, 170-184), as evidence to support its position that the specification must teach when to perform the method to enable the claims.

Von Herrath is cited as teaching that "[t]he type of autoreactive T-cell repertoire, the timing of antigen administration, and the choice of self-antigen are crucial features that must be understood if autoimmune diseases are to be controlled by immunization." (Paper 25, page 14, lines 16-18.) Von Herrath refers, at this citation, to the timing of delivery of a DNA vaccine to a patient *in vivo*, not delivery of APCs that are already transduced or transfected with a polynucleotide encoding an auto-antigen. Von Herrath teaches "an intriguing approach to the prevention of autoimmune disease, in which we use a DNA vaccine encoding a self-antigen to abrogate autoimmune disease." (Page 285, lines 13-15 of the Abstract.) Thus the claimed methods are completely different from the method taught by Von Herrath. Von Herrath's teachings regarding delivery of DNA to a patient are not relevant to the claimed methods of delivering APCs already transduced or transfected with a polynucleotide to an autoimmune disease patient.

Kristiansen is cited as teaching "future strategies should include a more specific approach to the possible involvement of CTLA4 in the pathogenetical process, e.g., by studying the CTLA4 expression during disease development-rather than studying only ligand-receptor interaction-in the rodent spontaneous disease models." (Paper 25, page 14, line 21 to page 15, line 1.) Kristiansen, at this passage, merely teaches that to determine what role CTLA4 plays in autoimmune disease it is important to determine whether CTLA4 expression is abnormal during development of the autoimmune disease. This teaching regarding CTLA4's potential role in

autoimmune disease also does not appear to be relevant to claims directed to a method of ablating auto-antigen-specific T cells in an autoimmune disease patient.

The Patent Office has not presented any evidence to support its assertion that the specification must teach when to administer APCs expressing an auto-antigen and Fas ligand to enable the claims.

(11) The Office Action asserts that the methods are not enabled because "the specification fails to teach the fate of the antigen presenting cells and viral vectors which express auto-antigens, whose consistent presence could either be a force to suppress antigen-specific activated T cells or a trigger to acerbate the autoimmune state." (Paper 25, page 15, lines 4-7.) The method claims have been amended to recite that the APCs administered to the autoimmune disease patient express Fas ligand. Thus the APCs deliver two signals to auto-antigen-specific T cells in the patient. First they stimulate the auto-antigen-specific T cells by engagement of the T cell receptor with the auto-antigen displayed on the APC surface. Second they ablate these T cells by engaging Fas on the auto-antigen-specific T cells with Fas ligand on the APCs. Thus if the APCs administered to the patient are consistently present, they will consistently stimulate and then ablate auto-antigen-specific T cells.

If a viral vector is used to transfer a polynucleotide to an APC, it also will not exacerbate the autoimmune disease by stimulating auto-antigen-specific T cells. The virus comprises polynucleotides that encode an auto-antigen. The auto-antigen-specific T cells are not activated by polynucleotides, they are activated by antigens or polypeptides. Thus the viruses will not stimulate the auto-antigen-specific T cells and the viruses will not exacerbate the autoimmune disease. Furthermore, the Office Action cites no evidence or sound scientific reasoning to

support its position that the viruses would be able to exacerbate the autoimmune disease condition.

(12) The Office Action asserts that the claims are not enabled because "it is unpredictable whether the method is able to ablate the autoreactive T cells in myasthenia gravis because [of] the immunological state of MG patients." (Paper 25, page 15, lines 12-14.) The Office Action cites Carrieri at Table 1 to support this statement. Carrieri teaches reasons why the thymus is believed to play a role in myasthenia gravis. It is true that myasthenia gravis patients may have thymic abnormalities. However, this does not change the simple fact an APC expressing an antigen recognized by an auto-antigen-specific T cell will activate that T cell, and that Fas ligand expressed on the APC will then ablate the activated T cell. Carrieri does not provide any reason to doubt this fact.

The claimed methods are adequately enabled by the teachings of the specification, applicant's declarations, and the knowledge of one of skill in the art at the time the application was filed. One of skill in the art could have practiced the claimed invention without recourse to undue experimentation.

Withdrawal of this rejection of claims 41-53 and 65 is respectfully requested.

The Rejection of Claims 54, 58, 59, 60, and 64 Under 35 U.S.C. § 102(e)

Claims 54, 58, 59, 60, and 64 are rejected under 35 U.S.C. § 102(e) as being anticipated by August et al. (U.S. Patent 5,633,234).

Claims 54, 58, and 60 have been canceled. Thus the rejection of these claims is rendered moot. Applicant respectfully traverses this rejection of amended claims 59 and 64.

Claims 59 and 64 depend from claim 55. Claim 55 is directed to viruses. The viruses infect human APCs and comprise "a polynucleotide encoding Fas ligand" and a "polynucleotide encoding a truncated form of FADD."

To reject claims as anticipated, each element of the claims must be found in a single prior art reference. "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). August, however, does not expressly or inherently teach each and every element recited in claim 55 and dependent claims 59 and 64.

August teaches vaccine compositions. The vaccine compositions comprise a viral vector and are used to treat an autoimmune disease patient. The viral vector transduces antigen presenting cells with an auto-antigen linked to endosomal/lysosomal targeting signals. The transduced antigen presenting cells are introduced into the patient and anergize auto-antigen specific T cells.

> [A] poor antigen presenting cell (that did not express any co-stimulatory signals) would either be induced to express MHC class II or would be transfected with the appropriate MHC class II genes. This cell would then be additionally transduced with the auto-antigen of interest, such as the acetylcholine receptor in the case of myasthenia gravis, linked to the endosomal/lysosomal targeting signal. Injection of these cells into the host would result in turning off T cell responses against the antigen.

Column 19, lines 41-50. August does not teach, however, polynucleotides encoding Fas ligand or FADD. Thus August cannot teach viral vectors comprising "a polynucleotide encoding Fas ligand" or a "polynucleotide encoding a truncated form of FADD" as recited in claim 55. August does not expressly or inherently teach each and every element recited in amended claim

55. Claims 59 and 64 are dependent from claim 55 and thus contain these elements. Thus August does not anticipate these claims.

Withdrawal of this rejection is respectfully requested.

The Rejection of Claims 58, 59, and 64 Under 35 U.S.C. § 102(e)

Claims 58, 59, and 64 are rejected under 35 U.S.C. § 102(e) as anticipated by Steinman *et al.* (U.S. Patent 6,300,090).

Claim 58 has been canceled. Thus the rejection of this claim has been rendered moot. Applicant respectfully traverses the rejection of claims 59 and 64.

Claims 59 and 64 depend from claim 55. Claim 55 is directed to viruses. The viruses infect human APCs and comprise "a polynucleotide encoding Fas ligand" and a "polynucleotide encoding a truncated form of FADD."

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Steinman, however, does not expressly or inherently teach each and every element as recited in claim 55 or in claims 59 and 64.

Steinman teaches viral vectors that deliver antigens to dendritic cells. The antigens include "autoantigens, . . . and any other antigens for which it is desired that they [be] presented by dendritic cells." (Column 9, lines 27-32.) The viral vectors may additionally contain a polynucleotide that encodes a protein to facilitate recognition of dendritic cells by the virus and entry of the virus into the dendritic cell. "[V]ectors may be targeted to dendritic cells by

modifying the viral vector to encode for a protein or parts thereof that is recognized by a receptor on dendritic cells, whereby occupation of the dendritic cell receptor by the vector will initiate endocytosis of the vector." (Column 8, lines 52-57.) Steinman does not, however, expressly or inherently teach Fas ligand or FADD. Thus Steinman cannot expressly or inherently teach viral vectors comprising "a polynucleotide encoding Fas ligand" and a "polynucleotide encoding a truncated form of FADD" as recited in claim 55. Claims 59 and 64 are dependent on claim 55 and therefore also contain these elements. Thus Steinman does not anticipate these claims either.

Withdrawal of this rejection is respectfully requested.

The Rejection of Claims 54, 58, 66, and 67 Under 35 U.S.C. § 103(a)

Claims 54, 58, 66, and 67 are rejected under 35 U.S.C. § 103(a) as being unpatentable over August *et al.* (U.S. Patent 5,633,234) in view of Atassi *et al.* (*Cri. Rev.* (1997) *17*, 481-495).

Claims 54 and 58 have been canceled. Thus the rejection of these claims is rendered moot. Applicant respectfully traverses the rejection of claims 66 and 67.

Claims 66 and 67 are directed to viruses and antigen presenting cells, respectively. The antigen presenting cells recited in claim 66 are transduced or transfected "with a polynucleotide sequence encoding a protein which is detrimental to activated T cell survival." The viruses recited in claim 67 comprise a "polynucleotide encoding Fas ligand" and a "polynucleotide encoding a truncated form of FADD."

To reject a claim as *prima facie* obvious three criteria must be met:

> First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable

> expectation of success. Finally, the prior art reference (or references when combined) **must teach or suggest all the claim limitations**.

MPEP § 1243; emphasis added. The *prima facie* case of obviousness must fail because the combination of August and Atassi does not teach or suggest all the limitations recited in claims 54 and 58.

As indicated above, August teaches viruses that transduce antigen presenting cells. The viruses comprise an "antigen of interest modified with the LAMP targeting signal." (Column 19, lines 8-9.) August also teaches antigen presenting cells transduced with the vector. "This [antigen presenting cell] would then be additionally transduced with the auto-antigen of interest . . . linked to the endosomal/lysosomal targeting signal." (Column 19, lines 45-48.) August does not, however, teach a polynucleotide encoding a protein detrimental to activated T cell survival, such as Fas ligand. August also does not teach a polynucleotide encoding a truncated form of FADD. Thus August does not teach or suggest viral vectors comprising "a polynucleotide encoding Fas ligand" and "a polynucleotide encoding a truncated form of FADD." August also does not teach or suggest an antigen presenting cell comprising "a polynucleotide sequence encoding a protein which is detrimental to activated T cell survival."

Atassi also does not teach or suggest polynucleotides that encode a protein detrimental to activated T cell survival, such as Fas ligand, or polynucleotides that encode a truncated form of FADD. Thus Atassi does not remedy these defects of August. Atassi mapped T and B cell epitopes of acetylcholine receptor α subunit involved in myasthenia gravis disease progression. Atassi teaches that these epitopes can be used to immunize animals to the acetylcholine receptor and suppress myasthenia gravis. The immunization composition includes acetylcholine receptor

epitope conjugated to monomethoxypolyethylene glycol. "It is known that injection of animals with conjugates of antigen to monomethoxypolyethylene glycol (mPEG) causes tolerance to subsequent immunization with the native antigen." (Page 489, column 1, lines 11-15.) Atassi does not, however, teach or suggest using any polynucleotide in the immunization composition including "a polynucleotide sequence encoding a protein which is detrimental to activated T cell survival," "a polynucleotide encoding Fas ligand," or "a polynucleotide encoding a truncated form of FADD." Thus Atassi cannot teach or suggest a virus comprising polynucleotides encoding Fas ligand and a truncated form of FADD. Atassi also cannot teach or suggest an antigen presenting cell transduced or transfected with a polynucleotide encoding a protein detrimental to activated T cell survival. Thus the combination of August and Atassi does not teach or suggest each and element recited in amended claims 66 and 67. The *prima facie* case of obviousness must fail. Withdrawal of this rejection is respectfully requested.

The Rejection of Claims 54-56, 58, 60-62, 66, and 67 Under 35 U.S.C. § 103(a)

Claims 54-56, 58, 60-62, 66, and 67 are rejected under 35 U.S.C. § 103(a) as being unpatentable over August *et al.* (U.S. Patent 5,633,234) in view of Bellgrau *et al.* (U.S. Patent 5,759,536).

Claims 54, 58, and 60-62 are canceled. Thus the rejection of these claims is rendered moot. The rejection of claims 55, 56, 66, and 67 is respectfully traversed. Claims 55, 56, and 66 are directed to antigen presenting cells. Claim 67 is directed to a virus. The rejection of each set of claims is addressed separately below.

Claims 55, 56, and 66

Claim 55 is the only independent claim of the set of claims directed to antigen presenting cells. Claim 55 recites antigen presenting cells transduced or transfected with "a polynucleotide encoding a protein comprising all or a portion of an auto-antigen to which the patient's antigen-specific T cell respond" and "a polynucleotide sequence encoding a protein which is detrimental to activated T cell survival."

To reject claims as *prima facie* obvious three criteria must be met:

> First, **there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings.** Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

MPEP § 1243, emphasis added. It is respectfully submitted the *prima facie* case of obviousness with respect to claims 55, 56, and 66 fails to meet the first criterion.

August teaches antigen presenting cells transduced with a vector to express an auto-antigen. (See column 19, lines 45-48.) August does not, however, teach a polynucleotide encoding a protein detrimental to activated T cell survival, such as Fas ligand.

Bellgrau teaches administration of Fas ligand to treat T-lymphocyte mediated autoimmune disease. Bellgrau teaches that Fas ligand causes "suppression of T-lymphocyte-mediated immune responses, including those directed against autologous tissue in autoimmune conditions." (Column 1, lines 14-16.) One of skill in the art would not, however, have modified August's transduced antigen presenting cells to further comprise a polynucleotide encoding Bellgrau's Fas ligand.

August teaches treatment of an autoimmune disease with antigen presenting cells transduced to express an auto-antigen but not co-stimulatory molecules. (See column 19, lines 41-43.) The antigen presenting cells do not express co-stimulatory molecules to prevent auto-antigen specific T cells from being activated. August teaches that the transduced APCs function by "turning off T cell responses against the antigen, based on the efficient presentation of peptide sequences on MHC class II molecules to T cell receptors on CD4+ T cells in the absence of the appropriate co-stimulatory signals (signals that are provided by effective antigen present cells)." (Column 19, lines 48-54.)

Bellgrau teaches that Fas ligand suppresses aberrant immune responses in auto-immune disease by killing activated T cells. Bellgrau teaches that "Fas ligand as an immunosuppressive agent is most active against a primed or activated immune system." (Column 5, lines 29-30.)

Thus, August teaches treatment of an auto-immune disease by administering antigen presenting cells that lack co-stimulatory molecules in order not to activate T cells. Bellgrau teaches administration of a polypeptide, Fas ligand, to suppress activated T cells. One of skill in the art would not have been motivated to include a molecule that kills activated T cells, Fas ligand, in antigen presenting cells specifically designed not to activate auto-antigen specific T cells. There would have been no motivation to combine the teaching of August with the teachings of Bellgrau. Thus the *prima facie* case of obviousness with respect to claim 55 and dependent claims 56 and 66 must fail.

Claim 67.

To establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981 (CCPA 1970).

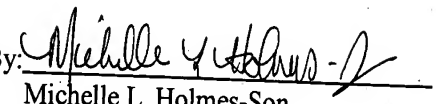
Claim 67 is directed to a virus. The virus infects human APCs and comprises "a polynucleotide encoding a truncated form of FADD."

August, as indicated above, teaches viruses that transduce APCs with an "antigen of interest modified with the LAMP targeting signal." (Column 19, lines 8-9.) August does not teach or suggest polynucleotides encoding FADD or viruses that comprise a polynucleotide comprising "a truncated form of FADD."

Bellgrau also does not teach a polynucleotide encoding a truncated form of FADD and thus fails to remedy this defect of August. Bellgrau teaches treatment of autoimmune disorders with "Fas ligand as an immunosuppressive agent." (Column 5, line 29.) Bellgrau teaches that Fas ligand can be administered by "implanting into patients, transfected cells capable of expressing and secreting a biologically active form of Fas ligand." (Column 6, lines 65-67.) Bellgrau does not, however, teach a polynucleotide encoding a truncated form of FADD or further transfectingv cells that express Fas ligand with a second polynucleotide encoding a truncated form of FADD. Thus the combination of August and Bellgrau does not teach or suggest "a polynucleotide encoding a truncated form of FADD" as recited in claim 67 and the *prima facie* case of obviousness must fail. Withdrawal of this rejection with respect to claim 67 is respectfully requested.

Respectfully submitted,

Date: March 4, 2003

By: 
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The Enhanced Immune Response to the HIV gp160/LAMP Chimeric Gene Product Targeted to the Lysosome Membrane Protein Trafficking Pathway*

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The lysosome-associated membrane proteins (LAMP), found in the outer membrane of lysosomes and also in a multilaminar compartment that contains major histocompatibility complex class II (MHC II) proteins, are directed to their localization by a cytoplasmic carboxyl-terminal sequence. Our studies of the immune response to LAMP-targeted proteins has led to the application of a HIV-1 gp160/LAMP chimeric gene as a novel means to enhance the MHC II presentation of gp160. Immunofluorescence microscopy confirmed that the gp160/LAMP protein had a cellular localization corresponding to that of lysosomes. Pulse-chase analysis confirmed that the rates of synthesis of gp160/LAMP and wild type gp160 were comparable and that both proteins were processed to gp120 at similar rates. However, the gp160/LAMP was degraded more rapidly than the wild type gp160. MHC II-mediated T cell proliferation assays performed with cloned human cell lines showed that gp160/LAMP stimulated greater responses than did the wild type gp160. Moreover, mice vaccinated with recombinant vaccinia expressing gp160/LAMP had greater gp160-specific lymphoproliferation responses and higher titers of anti-V3 loop antibodies than mice vaccinated with recombinant vaccinia expressing wild type gp160.

Much of the effort in developing an effective vaccine for HIV-1 has focused on the envelope protein. In addition to protein vaccines, some vaccine strategies have featured recombinant viruses that express the envelope protein (1–11), and other more recent studies have employed DNA immunization (12–17). Envelope-specific humoral and cell-mediated responses have been demonstrated by use of these approaches. However, one problem with DNA vaccines in general is that the vaccine antigen made in cells taking up the vector may not enter the major histocompatibility complex class II (MHC II)[1] antigen processing and presentation pathway, which conventionally operates only in professional antigen presenting cells (APC) that express MHC II molecules. This pathway is believed to be accessed primarily by the endocytosis or phagocytosis of extracellular proteins, with transport of the protein to endosomal/lysosomal compartments where it is proteolytically degraded, and the antigenic peptides loaded onto MHC II molecules for transport to the cell surface and presentation to $CD4^+$ T cells (18–20). Some endogenously synthesized membrane proteins, including the influenza hemagglutinin and HIV envelope protein, are also presented by MHC II molecules, presumably by endocytosis from the cell surface or other suggested pathways (21–24).

We have hypothesized that the targeting of recombinant antigens to the lysosome-associated membrane protein (LAMP) trafficking pathway will enhance the loading of endogenously synthesized antigen onto MHC II molecules and consequently elicit an enhanced $CD4^+$ helper T cell response with the resulting increase in both humoral and cell-mediated immunity. The basis of this approach is to create a chimeric antigen containing the endosomal/lysosomal localization signal from the LAMP family of lysosomal membrane proteins (25, 26). The LAMP molecules are type I transmembrane proteins that contain a 24-amino acid transmembrane domain and an 11-amino acid cytoplasmic tail with a carboxyl-terminal YQTI sequence that, in the context of the LAMP cytoplasmic domain, is necessary and sufficient to target recombinant proteins through a vesicular pathway to lysosomes (27). Recent evidence has indicated that at some point in the trafficking of the LAMP protein it enters a compartment that also contains MHC II molecules. Several electron microscopy and cell fractionation studies have shown co-localization of LAMP with MHC II molecules (28–31), and a multilaminar prelysosomal compartment containing LAMP and MHC II molecules, termed MIIC by some, has been implicated as the site of antigen processing and peptide loading onto MHC II molecules (32–35). We propose that the co-localization of LAMP with MHC II proteins, or possibly the more efficient processing and delivery of antigen peptides to MHC II as a result of LAMP-mediated targeting, may provide the basis for an enhanced immune response to the LAMP-targeted as compared with the wild type antigen. The enhancement of both cell-mediated and humoral immune responses by LAMP targeting of antigen has been demonstrated by Wu *et al.* (36), with a recombinant vaccinia

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[1] The abbreviations used are: MHC II, major histocompatibility complex class II antigens; LAMP, lysosome-associated membrane protein; APC, antigen presenting cells; m.o.i., multiplicity of infection; B-LCL, EBV-transformed B lymphoblastoid cell lines; PBS, phosphate buffered saline solution; BCM, B cell medium; TCR, T cell receptor; HA, hemagglutinin; pfu, plaque-forming units; MEM, minimal essential medium; HIV, human immunodificiency virus; EBV, Epstein-Barr virus.

virus-expressed HPV-16 E7 cytoplasmic/nuclear antigen. In this study, LAMP targeting was shown to enhance $CD4^+$ T cell responses to the E7 antigen in *in vitro* systems and to increase both humoral and cell mediated anti-E7 responses in vaccinated mice. In addition, it was shown that vaccination of mice with LAMP-targeted E7 antigen elicited a much stronger immune response to tumor cells bearing the E7 antigen than did vaccination with wild type E7 (37).

In a previous report, we described the LAMP-directed sorting of the HIV gp120 envelope protein to lysosomes through the synthesis of a CD4/LAMP chimeric protein containing the LAMP lysosomal targeting sequence (38). The hypothesis underlying this study was that, when both CD4/LAMP and soluble gp120 are expressed in the same cell, the CD4/LAMP molecule would bind gp120 and direct the viral protein to the MHC II processing pathway. The feasibility of direct targeting was also explored. In this present study, we have further characterized the response to LAMP targeting of the HIV gp160 antigen by placing the LAMP targeting sequence directly into the gp160 envelope protein. We have demonstrated the lysosomal localization of this chimeric gp160/LAMP and shown that this localization is associated with a more rapid degradation of the protein. Cytolytic and proliferation assays were performed using several lines of cloned human $CD4^+$ cytotoxic T cells. The gp160/LAMP construct was shown to stimulate greater proliferation responses of the T cell clones *versus* the wild type gp160. Moreover, immunization of mice with recombinant vaccinia expressing the gp160/LAMP chimeric protein resulted in an enhanced induction of helper T cell responses as evidenced by increases in antigen-specific lymphoproliferation responses and antibody levels than did vaccination with recombinant vaccinia expressing the wild type gp160.

EXPERIMENTAL PROCEDURES

HIV gp160 and LAMP Gene Constructs and Recombinant Vaccinia—The HIV-1 gp160 envelope gene used in these studies was derived from HIV-1 LAI (39). The vaccinia vector vPE16 and plasmid pPE15 both contain the HIV-1 LAI gp160 gene and have been described previously (40, 41). The gp160 gene encoded by both the vPE16 and pPE15 was engineered to contain silent mutations that remove two cryptic vaccinia early transcription termination signals. These constructs express full-length envelope protein, and the silent mutations result in increased expression of gp160 protein in vaccinia-infected cells. The gp160/LAMP chimeric gene was constructed by first amplifying DNA encoding the transmembrane and cytoplasmic domains of murine LAMP-1 by polymerase chain reaction. The product was engineered to contain *Eco*RI and *Xho*I sites at the 5′ and 3′ ends. The sense primer, 5′-GGGGGAATTCTTGATCCCCATTGCTGTGGGC-3′ (*Eco*RI site in bold), was annealed to mLAMP-1 nucleotides encoding Leu^{348}-Gly^{354}. The antisense primer, 5′-AAAACTCGAGCTAGATGGTCTGATAGCCGGC-3′ (*Xho*I site in bold, stop code underlined), was annealed to LAMP-1 nucleotides encoding amino acids Ala^{377}–Ile^{382}. The 120-base pair product was cloned into the *Eco*RI and *Xho*I sites of the vector pCDNA1 (Invitrogen, San Diego, CA) to form pCDNA1/L1 and confirmed by DNA sequencing. The plasmid pPE15 was cut with *Hin*dIII and *Eco*RI yielding a 1.2-kilobase pair fragment encoding the amino-terminal extracellular portion of HIV gp160. This fragment was then cloned into pCDNA1/L1 at *Hin*dIII and *Eco*RI sites to form pCDNA1/GP160(5′)/L1. This plasmid was cut with *Bam*HI and *Xho*I and the 1.32-kilobase pair fragment was cloned into the *Bam*HI and *Xho*I sites of pGEM7Z (Promega, Madison, WI) to form pGEM7Z/GP160(5′)/L1. The DNA encoding the remainder of the extracellular portion of HIV gp160 was amplified by polymerase chain reaction using the *Pfu* polymerase (Stratagene, La Jolla, CA) from just upstream of the internal *Eco*RI site and was engineered to contain an additional *Eco*RI site at the 3′ end just 5′ to the start of its transmembrane domain. The sense primer, 5′-GGAGGGGACCCAGAAATTGTAAGCG-3′ annealed to nucleotides 1095 to 1120. The antisense primer, 5′-TTTTTGAATTCTTTTATATACCACAGCCAATTTGT-3′ annealed to nucleotides 2011 to 2035 (*Eco*RI site in bold). This fragment was cloned into the *Eco*RI site of pGEM7Z/GP160(5′)/L1 to form pGEM7Z/160/L1. This HIV gp160/LAMP chimera was cloned into the vaccinia recombination vector pSC11MCS1 (41) using the *Sal*I and *Sph*I restriction enzymes to form the recombinant vaccinia virus vTA160/L1 as described (42–44). The recombinant vaccinia vHA/LAMP expressing the influenza hemagglutinin antigen/LAMP chimera has been described elsewhere,[2] and was used in these studies for a negative control of a vaccinia-expressed LAMP-targeted irrelevant antigen. All of the recombinant vaccinias used in this study were derived from the WR strain.

Immunofluorescence—Human embryonic kidney 293S cells (2 ml of 2.5×10^5 cells/ml) in MEM-10 (MEM, 10% HyClone Fetal Clone III serum product, 100 units/ml penicillin G, and 100 μg/ml streptomycin) were plated in a six-well tissue culture plate containing sterile glass coverslips. The cells were incubated overnight at 37 °C in a 5% CO_2 incubator. The medium was aspirated and 0.5 ml of complete MEM-2.5 (same as MEM-10 except it contains 2.5% serum product) was added containing the recombinant vaccinia vTA160/L1 or vPE16 at a multiplicity of infection (m.o.i.) of 1. The cells were incubated for 1 h, then overlaid with 1.5 ml of complete MEM-2.5 and incubated overnight. Subsequently, the cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. After washing with PBS, PBS containing 4% normal goat serum and 0.1% saponin was added to block and permeabilize the cells. The cells were then incubated for 45 min with monoclonal antibody 902 (45) supernatant medium containing 0.1% saponin and 4% normal goat serum. After washing twice with PBS, the cells were incubated with 1 ml of PBS containing 0.1% saponin and 10 μg/ml Texas Red-conjugated goat anti mouse IgG (Jackson, West Grove, PA). The cells were then washed twice in PBS and the coverslip inverted onto a microscope slide with a drop of mounting medium. The slides were observed under a Zeiss Axiophot and exposures were adjusted manually using Kodak TMAX 400 film.

Pulse-Chase and Immunoprecipitation—Separate culture tubes for each time point of the pulse-chase protocol contained 3×10^6 human EBV-transformed B lymphoblastoid cells, clone 81 EBV, at log phase growth in 1 ml of B cell medium (BCM) (RPMI 1640, 10% fetal bovine serum, 200 units/ml penicillin G, 200 μg/ml streptomycin, and 4 mM L-glutamine). The cells were infected with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP at a m.o.i. of 5, and after 2 h, washed three times with 5 ml of methionine-free RPMI 1640 and then incubated in 1 ml of methionine-free BCM for 20 min. The cells were then pulsed with 150 μCi of [^{35}S]methionine (Amersham Corp.) for 10 min. BCM (10 ml) was immediately added as chase medium, and the cells were washed twice with 5 ml of BCM. The cells were resuspended in 5 ml of BCM and placed into a T-25 flask for the chase period incubation. For chase times shorter than 30 min, the wash steps were omitted and the cells were incubated in the 10 ml of BCM. At the end of each chase time, the cells were pelleted by centrifugation, washed twice with ice-cold PBS, and resuspended in 0.5 ml of ice-cold lysis buffer (90 mM KCl, 5 mM $MgCl_2$, 1 mM benzamidine, 10 mM HEPES, 1 mM dithioerythritol, 0.5% Nonidet P-40, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, and 1 μg/ml aprotonin). After incubation on ice for 30 min, nuclei were removed by centrifugation for 10 min in a microcentrifuge. The cell lysates were precleared overnight with 3 μl of normal human serum (Jackson) and 150 μl of protein G-Sepharose (Pharmacia Biotech Inc.) suspended in an equal volume of Sepharose wash buffer (PBS, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mM dithiothreitol). The envelope protein was precipitated from the precleared lysates with 3 μl of heat-inactivated human antisera from HIV-positive donors and 30 μl of the protein G-Sepharose suspension. The antigen-antibody-Sepharose pellet was boiled for 5 min in 2 × reducing sample buffer. The samples were resolved on SDS 9% polyacrylamide gels, and the dried gels were exposed to a Fuji BAS-IIIs phosphor image screen for 20 h. The screen images were captured with a Fuji BAS1000 phosphor image scanner and MACBAS version 2.0 software.

T Cell and B Cell Lines—The human $CD4^+$ T cell lines and autologous EBV-transformed B lymphoblastoid cell lines (B-LCL) were previously described (46–48). All T cell clones were isolated from HIV-1 seronegative individuals who participated in various HIV-1 candidate vaccine programs or by *in vitro* stimulation of T cells with gp120-pulsed monocytes.

Cloned Human T Cell Cytolytic Assays—B-LCL target cells at a concentration of 1×10^6 cells/ml in BCM, were infected overnight with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP at a m.o.i. of 5. [^{51}Cr]Na_2CrO_4 (Amersham; 50 μCi/ml) was added to the target

[2] K. Staveley-O'Carroll, B. Purow, Y. Chen, A. L. Ruff, S. R. Schell, H. Levitsky, D. Pardoll, J. T. August, and F. G. Guarnieri, unpublished observations.

cells and incubated for 2 h. The cells were then washed three times with wash medium (PBS with 1% Fetal Clone III serum product), resuspended in 1 ml of BCM, counted for viable cells, and diluted to 1×10^5 cells/ml in BCM. Effector cells (autologous non-transformed human T cell clones) were counted for viability and resuspended at 1×10^6, 3×10^5, and 1×10^5 cells/ml in BCM. In V-bottom 96-well plates, 100 μl of the target cell suspension was plated in triplicate with the addition of: 1) 100 μl of each effector T cell dilution (effector/target ratios of 1:1, 3:1, and 10:1), or 2) 100 μl of 1% Nonidet P-40 (maximum lysis), or 3) 100 μl of BCM (nonspecific lysis). The plates were centrifuged for 5 min at 1200 RPM then incubated for 4 h at 37 °C in 5% CO_2. The plates were centrifuged again for 5 min, and 100 μl of supernatant was collected. Supernatant total counts were measured in a γ counter. Specific lysis was measured as % specific lysis = (experimental lysis − nonspecific lysis/maximum lysis − nonspecific lysis) × 100.

Cloned Human T Cell Proliferation Assays—B-LCL stimulator cells were pulsed with antigen by overnight infection with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP at a m.o.i. of 5 in 1×10^6 cells/ml in BCM. Proliferation of stimulator cells and vaccinia was blocked by treatment with either 5000-rad γ irradiation or psoralen treatment (incubation in 10 μg/ml of psoralen for 15 min followed by long wave UV light for 5 min). These infected stimulator cells were washed three times with wash medium and resuspended to 5×10^4 cells/ml in BCM. Infected cells were combined with non-infected cells at ratios of 1:0, 1:2, 1:4, and 1:10. Responder autologous T cells were resuspended to 5×10^5 cells/ml in BCM. The T cell suspension, 100 μl, was combined with 100 μl of each stimulator cell dilution in triplicate in U-bottom 96-well plates. After incubation for 72 h, 1 μCi of [^{3}H]thymidine (Amersham) was added to each well and cells were harvested 24 h later with a Packard Micromate cell harvester. [^{3}H]Thymidine incorporation into DNA was measured on a Packard Matrix 96 direct β counter.

Lymphoproliferation Assay of T Cells from Vaccinated Mice—BALB/c mice (Charles River Laboratories, Wilmington, MA), 6–8 weeks old, were immunized by footpad injection with 1×10^6 plaque-forming units (pfu)/mouse of purified recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP diluted in PBS. Two mice were used for each recombinant vaccinia group. The mice were boosted 2 weeks later using the same immunization protocol. After 3 weeks, the mice were euthanized by CO_2 asphyxiation, and the regional lymph nodes from both mice in each group were removed, pooled, crushed to form a single cell suspension, and passed through a 200-μM nylon screen. T cells were enriched from this suspension by non-adherence to nylon wool as described (49). Peptides HP19 (AA 322–336, FVTIGKIGNMRQAHC) and HP33 (AA 485–499, KYKVVKIEPLGVAPT) (Johns Hopkins University Protein Laboratory, Baltimore, MD), BALB/c MHC II gp160 peptide epitopes (50), were diluted in C-RPMI (RPMI 1640, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol) and dispensed into the wells of a 96-well U-bottom plate so that the final concentration after the addition of cells was 10, 3.15, and 0.99 μg/ml. The plate also contained negative and positive control wells with no peptide antigen or 2 μg/ml phytohemagglutinin (Sigma), respectively. Psoralen-treated, naive syngeneic splenocytes were added at 1×10^5 cells/well in C-RPMI. The previously enriched responder T cells were suspended in C-RPMI and plated at 3×10^5 cells/well in triplicate. The plate was incubated for 5 days in a humidified 37 °C incubator with 5% CO_2. After the incubation period, 1 μCi of [^{3}H]thymidine (Amersham) was added to each well and cells were harvested 24 h later with a Packard Micromate cell harvester. [^{3}H]Thymidine incorporation into DNA was measured on a Packard Matrix 96 direct β counter.

Antibody Responses of Vaccinated Mice—BALB/c mice (Charles Rivers, Wilmington, MA), 6–8 weeks old, were immunized by tail vein injection with 5×10^5 pfu/mouse of purified recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP diluted in PBS. Four mice were used for each recombinant vaccinia group. The mice were boosted twice at 2-week intervals by the same immunization protocol. Blood (200 μl) was collected by tail bleed 13 days after each immunization and centrifuged to remove cells, and the serum collected and stored at 4 °C for immediate use. V-3 loop peptide (AA 305–329, CNNTRKSIRIQRGPGRAFVTIGKIG) was diluted in enzyme-linked immunosorbent assay protein dilution buffer (50 mM Tris, pH 9.5) at a concentration of 38 μg/ml, and 100 μl of the solution was added to each well of an enzyme immunoassay/radioimmunoassay high binding 96-well plate (Costar, Cambridge, MA). After overnight incubation at 4 °C, the peptide solution was removed and 200 μl of blocking buffer (PBS with 0.05% Tween 20, 4% bovine serum albumin, and 4% normal goat serum) was added. The plates were incubated overnight at 4 °C and then washed with PBS containing 0.05% Tween 20 wash buffer. Serum samples were prepared in five serial dilutions from 10^{-1} to 10^{-5} in blocking buffer, and 100 μl of each dilution was added to the blocked plate. The plate was incubated overnight at 4 °C. Horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson) was diluted in blocking buffer to 0.5 μg/ml, and 100 μl was added to each well after washing the plate. The plates were incubated at room temperature for 4 h and then washed. Turbo TMB substrate solution (Pierce, 100 μl/well) was added to each well and incubated for 10 min at room temperature. The reaction was stopped by adding 100 μl of 1 M sulfuric acid, and absorbance at 450 nm was measured in a Cambridge Technology model 750 plate reader.

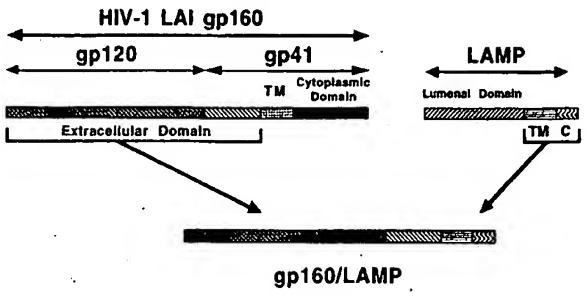

FIG. 1. **Construction of the gp160/LAMP chimera.** The HIV-1 LAI gp160 gene was engineered to contain silent mutations that remove two cryptic vaccinia early transcription termination signals. The gene sequence encoding all of gp120 and the entire extracellular domain of gp41 was linked to the gene sequence encoding the transmembrane (*TM*)/cytoplasmic (*C*) domains of the murine lysosomal membrane glycoprotein, LAMP-1. The chimeric gene, gp160/LAMP, was cloned into vaccinia to form the recombinant vaccinia vTA160/L1.

RESULTS

Construction of the gp160/LAMP Chimera—The gp160/LAMP chimeric gene construct was prepared by ligating the sequence encoding the entire extracellular domain of HIV-1 LAI gp160 to the sequence encoding the transmembrane/cytoplasmic domains of the murine lysosomal membrane glycoprotein, LAMP-1, as described in the methods (Fig. 1). DNA sequence analysis was performed to verify gene fidelity. The chimeric gene, gp160/LAMP, was then cloned into vaccinia to form the recombinant vaccinia vTA160/L1.

Protein Expression and Lysosomal Targeting—Other reports have shown the localization of LAMP-targeted chimeric proteins to the lysosomal membrane (27). We have used a similar immunofluorescence experiment in this study to verify the expression and lysosomal targeting of the chimeric gp160/LAMP protein (Fig. 2). Human embryonic kidney 293S cells were infected overnight with recombinant vaccinia vTA160/L1 or vPE16 expressing the chimeric or wild type protein, respectively, and stained the next day with a monoclonal antibody specific for gp120. The LAMP-targeted and wild type proteins were found to have distinctly different cellular staining patterns. Cells that expressed the wild type gp160 (*panel B*) had the expected reticular staining pattern. In contrast, cells that expressed the gp160/LAMP chimera (*panel A*) had a vesicular staining pattern characteristic of the lysosomal localization of LAMP and LAMP chimeras (25, 27).

Pulse-Chase Analysis of Protein Synthesis and Degradation—The biochemical properties of gp160/LAMP were further characterized by a kinetic pulse-chase labeling analysis of the synthesis and degradation of the LAMP-targeted and wild type gp160 (Fig. 3). B-LCL cells were infected with recombinant vaccinia, vTA160/L1, vPE16, or vHA/LAMP, labeled with [^{35}S]methionine for 10 min, and chased with excess unlabeled methionine for the times indicated in the figure. The HIV envelope proteins were immunoprecipitated with human HIV antiserum and resolved by SDS-polyacrylamide gel electrophoresis. The wild type gp160 and gp160/LAMP can be distinguished because the gp160/LAMP has a lower molecular weight than the wild type protein; the LAMP transmembrane/

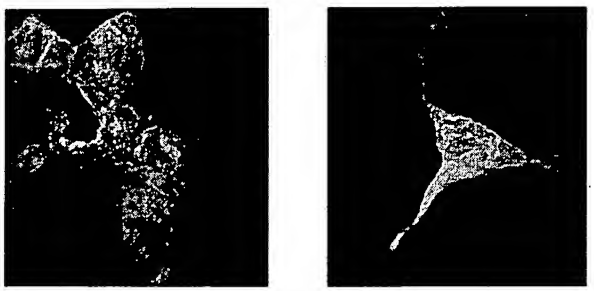

FIG. 2. **Immunofluorescence localization of gp160 and gp160/LAMP.** Human embryonic kidney 293S fibroblast cells were infected with recombinant vaccinia vTA160/L1 or vPE16 overnight and stained with mouse anti-gp120 monoclonal antibody 902 (45). Cells infected with vTA160/L1 have a granular staining pattern consistent with lysosomal localization of the gp160/LAMP protein (*A*). Cells infected with vPE16 have the expected reticular staining pattern of the wild type gp160 (*B*).

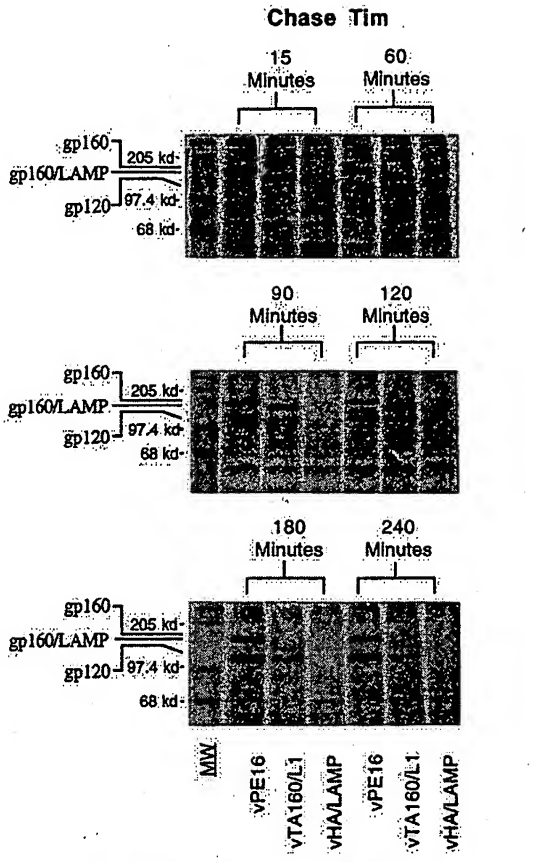

FIG. 3. **Pulse-chase, [^{35}S]methionine labeling, immunoprecipitation, and phosphor image of HIV envelope proteins expressed by recombinant vaccinia-infected cells.** 81 EBV B-LCL cells were infected with the recombinant vaccinia virus vPE16, vTA160/L1, or vHA/LAMP for 2 h. These cells were then pulsed for 10 min with [^{35}S]methionine and chased with excess unlabeled methionine for the time indicated. After the chase time, lysates were prepared and the gp160 proteins immunoprecipitated with human antisera from HIV-positive donors and protein G-Sepharose. The samples were resolved by SDS-polyacrylamide gel electrophoresis, and the dried gels were exposed to a phosphor image plate for 20 h. The chase times and recombinant vaccinia for each lane are given in the figure, and the location of the wild type gp160, gp160/LAMP, and gp120 protein bands are indicated at the *left* of each panel. Molecular weight markers (*MW*) are as shown. The rate of synthesis of the wild type gp160 and gp160/LAMP proteins was similar as indicated by the similar intensities of the wild type gp160 (160 kDa) and gp160/LAMP (149 kDa) protein bands at the 15-min chase point. Processing of gp160 to yield gp120 (120 kDa) was apparent at 60 min. At 90 min the intensities of the LAMP-targeted gp160 and gp120 proteins began to fade, and by 240 min, the LAMP-targeted proteins had almost completely disappeared. In contrast, the intensity of wild type gp160 and gp120 protein bands appeared unchanged throughout the 240-min experiment.

cytoplasmic domains of the gp160/LAMP being 101 amino acids smaller than the transmembrane/cytoplasmic domains of the gp41 subunit. At 15 min, the intensities of the wild type gp160 and the gp160/LAMP protein bands was equivalent, as confirmed by densitometry, indicating that the rate of synthesis of these two proteins was essentially equal. Processing of the proteins to yield gp120 was first apparent at 60 min and more so at 90 min. The gp160 protein band was apparent at every time point throughout the experiment consistent with a previous study showing that only a fraction of the gp160 protein is processed to the constituent gp120 and gp41 subunits (51). Beginning at 120 min, the intensities of the LAMP-targeted gp160 and gp120 protein bands were decreased relative to the 15-min time point and to the wild type proteins, and at 240 min, the LAMP-targeted gp160 and gp120 proteins had almost completely disappeared. In contrast, there was no obvious degradation of the wild type gp160 and gp120 proteins. No protein of similar molecular weight to the wild type gp160, gp160/LAMP, or gp120 was apparent in any of the lanes from cells infected with the negative control vHA/LAMP construct.

Cloned T Cell Cytolytic and Proliferation Assays of MHC II Presentation—MHC II presentation was analyzed by use of a cytolytic assay, which takes advantage of the fact that some $CD4^+$ T cells have cytolytic activity (46–48). Autologous target B-LCL cells were infected overnight with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP, labeled with [^{51}Cr]sodium chromate, and mixed with a $CD4^+$ cytotoxic T cell clone. Because the effector cells are $CD4^+$, they are MHC II-restricted and lyse only those target cells presenting gp120 peptides in MHC II molecules. Two different T cell clones, Een 217 and 11.8, were tested. These clones were derived from different donors and recognize different gp120 peptides presented by distinct MHC II alleles. In each case, lysis of target cells expressing gp160/LAMP was greater than or equal to lysis of target cells expressing wild type gp160 (Fig. 4). These results indicate that gp160/LAMP is processed for MHC II-restricted recognition at least as well as the wild type gp160. The difference in specific lysis was small owing to the high sensitivity of this assay and the end point being limited to 100% specific lysis. It is therefore possible that the cytolytic assay underestimates the real differences in processing of the wild type and LAMP-targeted antigens.

A more relevant measure of the ability of the LAMP-targeted antigen construct to enhance MHC II presentation involves the ability of APC to induce proliferation of $CD4^+$ T cells. MHC II-restricted $CD4^+$ T cell proliferation responses were measured by overnight infection of antigen presenting stimulator cells, autologous B-LCL, with the recombinant vaccinia vPE16, vTA160/L1, or vHA/LAMP, blocking the proliferation of the cells and vaccinia with psoralen treatment or γ irradiation, and mixing them with a responding $CD4^+$ T cell clone specific for a gp120 epitope. Three T cell clones derived from different donors were tested. These clones each had a different MHC II restriction and gp120 epitope specificity. All showed a marked increase in their proliferation response to the lysosome-targeted gp160/LAMP antigen *versus* the wild type gp160 (Fig. 5).

Mouse Vaccination and Immune Responses—The potential utility of the LAMP targeting of gp160 to enhance helper T cell responses in a vaccine was assessed by analysis of the humoral and lymphoproliferative response of vaccinated mice. For assay

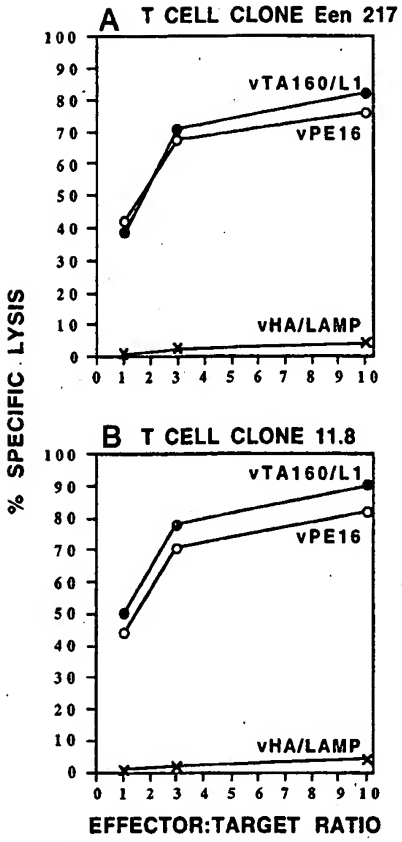

FIG. 4. **Lysis of recombinant vaccinia-infected cells by CD4⁺ cytolytic T cells.** These studies utilized cloned human CD4⁺ cytotoxic T cells specific for gp120 epitopes as effector cells and autologous EBV-transformed B-LCL cells as target cells. Target cells were infected overnight with vPE16, vTA160/L1, or vHA/LAMP and labeled with ^{51}Cr for 2 h. These cells were mixed with the effector T cells at effector to target ratios of 1:1, 3:1, and 10:1. After incubating for 4 h, 100 μl of the culture supernatants was removed and [^{51}Cr] release measured by a γ counter. The data are plotted as % specific lysis *versus* effector: target cell ratio. Two effector cell lines were tested: T cell clone Een 217 with autologous target cell population B-LCL Laz 509 (*A*) and T cell clone 11.8 with autologous target cell population B-LCL 11EBV (*B*).

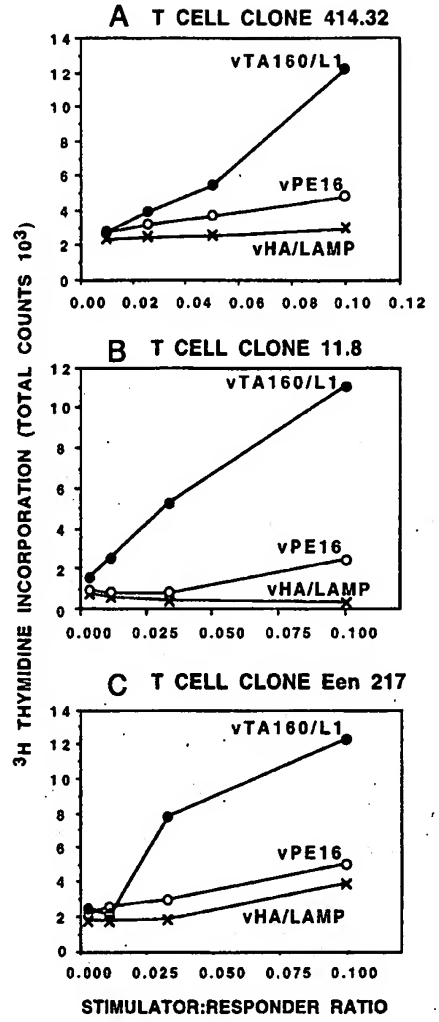

FIG. 5. **T cell proliferation induced by recombinant vaccinia-infected stimulator cells.** These studies utilized cloned human CD4⁺ T cells specific for gp120 epitopes as responder and autologous EBV-transformed B cells as antigen presenting stimulator cells. Stimulator cells were infected overnight with vPE16, vTA160/L1, or vHA/LAMP, and proliferation was blocked by psoralen treatment or γ irradiation. The stimulator cells were then mixed with the responding T cell clone at stimulator to responder ratios indicated in the figure. After incubation for 72 h, the cells were pulsed with [^{3}H]thymidine and harvested 24 h later. Proliferation was measured by [^{3}H]thymidine incorporation into DNA. The data are plotted as total counts incorporated *versus* stimulator cell:responder cell ratio. Three responder cell lines were tested: T cell clone 414.32 with autologous stimulator cell population B-LCL 414EBV (*A*), T cell clone 11.8 with autologous stimulator cell population B-LCL 11EBV (*B*), and T cell clone Een 217 with autologous stimulator cell population B-LCL Laz 509 (*C*).

of the humoral response, BALB/c mice were vaccinated three times at 2-week intervals by tail vein injection with purified recombinant vaccinia vTA160/L1, vPE16, vHA/LAMP, or PBS (vehicle control). Serum samples were collected 13 days after each immunization, and antibodies to the V3 loop were measured by peptide enzyme-linked immunosorbent assay (Fig. 6). The anti-V3 loop antibody responses of mice vaccinated with vPE16 were at or below the background levels of the PBS and vHA/LAMP-negative controls. In contrast, in mice vaccinated with vTA160/L1, the LAMP targeted gp160 generated robust anti-V3 loop antibody titers.

The lymphoproliferation response was measured with T cells from BALB/c mice vaccinated twice with a 2-week interval by footpad injection with the recombinant vaccinia vTA160/L1, vPE16, or vHA/LAMP. Three weeks after the last immunization, T cells were isolated from the regional lymph nodes pooled from the two mice in each group. These cells were mixed with stimulator cells (naive splenocytes) and two peptides, HP19 and HP33, which are BALB/c MHC II gp160 peptide epitopes. The proliferation of the T cells from mice that received the LAMP-targeted gp160 was several times greater than that of T cells from mice that received the wild type gp160 (Fig. 7); the response to the wild type gp160 remained at background level.

DISCUSSION

Infection of antigen presenting cells or mice with the recombinant vaccinia virus, vTA160/L1, expressing HIV gp160 as a chimeric protein containing the LAMP lysosomal targeting sequence, was found to elicit increased responses of cloned CD4⁺ T cells *in vitro* and increased lymphoproliferation and anti-V3 loop antibody titers *in vivo*, as compared with the recombinant vaccinia virus vPE16 expressing wild type gp160. The cytolytic and proliferation responses of the cloned human CD4⁺ T cells suggest that these effects result from an enhanced MHC II processing and presentation of the chimeric gp160/LAMP, evidently as a consequence of its trafficking through the LAMP protein pathway. This model was dependent upon the demon-

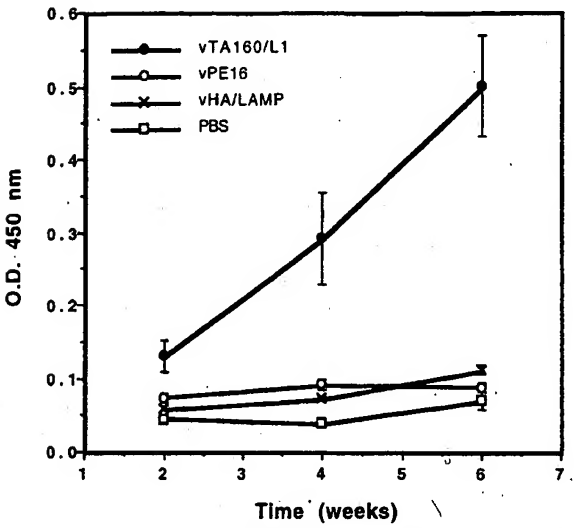

FIG. 6. **Anti-V3 loop antibody responses of vaccinated BALB/c mice.** Mice were vaccinated by tail vein injection with 5×10^5 pfu of the recombinant vaccinia vTA160/L1, vPE16, vHA/LAMP, or PBS control three times at 2-week intervals. Serum samples were collected 13 days after each immunization. The serum dilution in the experiment for this figure was 1:1000, which was the lowest serum dilution at which the mean of the absorbances of the vehicle controls was less than 0.1 at all time points. The value for each group is the mean of the absorbances from four mice. The data are plotted as $A_{450\,nm}$ *versus* time in weeks ± standard error.

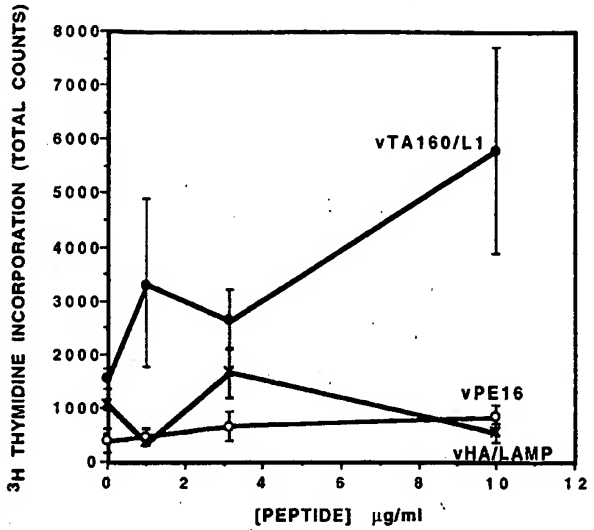

FIG. 7. **Lymphoproliferation responses of T cells from vaccinated BALB/c mice.** Mice were vaccinated by footpad injection with 1×10^6 pfu of the recombinant vaccinia vTA160/L1, vPE16, or vHA/LAMP two times with a 2-week interval. Lymph nodes were removed 3 weeks after the second immunization, and T cells were isolated from the pooled lymph nodes of each group. These cells were mixed with naive splenocytes as stimulator cells and peptides HP19 and HP33, BALB/c MHC II gp160 peptide epitopes, as indicated in the figure. After incubation for 5 days, the cells were pulsed with [^{3}H]thymidine and harvested 24 h later. Proliferation was measured by [^{3}H]thymidine incorporation into DNA. The data are plotted as total counts incorporated *versus* peptide concentration.

stration that the protein was indeed delivered to lysosomes and that the increased response to the LAMP-targeted protein was not due to a greater production of the chimeric protein. The steady-state cellular localization of the gp160/LAMP chimera in lysosomes was demonstrated by immunofluorescence microscopy, as described previously for LAMP proteins themselves (25) and other LAMP chimeras (27). Kinetic pulse-chase experiments confirmed that the gp160/LAMP protein was synthesized at a rate similar to that of the wild type gp160, thus indicating that the increased immune response to the LAMP-targeted protein cannot be attributed to an increased rate of synthesis of the protein. Most importantly, the pulse-chase analysis demonstrated that the chimeric protein was more rapidly degraded than the wild type gp160, as would be expected for a molecule delivered to lysosomes. The degradation of LAMP targeted gp160 (and gp120) became evident after 90 min, consistent with the results of a previous kinetic analysis of the biosynthesis and localization of LAMP-1 in mouse embryo 3T3 cells, which showed that approximately 1 h was required for delivery of newly synthesized protein to lysosomes (52). In contrast, the apparent concentration of the wild type gp160 (and gp120), remained constant throughout the 4-h experiment. It is also noteworthy that despite its difference in structure and vesicular trafficking, the gp160/LAMP protein was processed to yield the gp120 subunit at a rate similar to the wild type gp160 and comparable to that shown by others for the gp160 in HIV-1 pNL4–3-infected human lymphocytic leukemia cells (51). It is intriguing, however, that there are otherwise significant differences between the fate of gp160 synthesized in the HIV virus-infected lymphocytic leukemia cells as compared with that synthesized in vaccinia-infected B-LCL cells. Specifically, the gp160 made in HIV-infected cells was rapidly degraded during the first 4 h of synthesis in a process inhibited by NH_4Cl, suggesting that the degradation occurred in an acidic compartment with other evidence that this occurred in the lysosome (51). In contrast, the wild type gp160 made in vaccinia virus-infected cells was stable through 4 h. These findings raise the possibility that the vesicular trafficking and processing of HIV virus-expressed gp160 and the vaccinia-expressed gp160 differ or that the differences are a function of the host cells.

The *in vitro* assays, target cell cytolysis and T cell proliferation, used in this study to analyze MHC II presentation of antigen both utilized cloned human $CD4^+$ cytotoxic T cells specific for gp120 epitopes. The data showed that LAMP targeting of the envelope protein enhanced both of these MHC II-mediated responses. The most significant response occurred with the T cell proliferation assays, which compared with the cytolytic assays, have the advantage of not having a limited end point. Here, stimulator cells expressing the LAMP-targeted gp160 elicited proliferation responses that were severalfold greater than the responses elicited by the cells expressing wild type gp160. This is despite the fact that wild type gp160 is presented in MHC II (21, 22), and elicits a $CD4^+$ T cell proliferation response. Thus, LAMP targeting appears to be superior to the normal gp160 processing pathway for MHC II presentation. In this context it should also be noted that the direct targeting of the gp160 to the lysosomal pathway is more efficient than the use of a CD4/LAMP chimeric molecule to direct the associated gp120 to the MHC II antigen processing pathway (38). It may be that the CD4/LAMP molecule itself is not as efficiently directed to the lysosome or that the kinetics of the binding of gp120 to CD4/LAMP limit the processing or presentation of gp120 for MHC II by this indirect mechanism. In the cytolytic assays of this study, although the difference in specific lysis between the gp160/LAMP and wild type gp160 groups was small, approximately 9%, the LAMP-targeted antigen elicited consistently greater cytolytic responses. The lower difference in response between the targeted and wild type antigen in the cytolytic as compared with the proliferation assays is attributed to the high sensitivity and limited end point of the cytolytic assay.

The extent of the immune response of mice vaccinated with

the LAMP-targeted gp160 as compared with the absence of response to the wild type gp160 was remarkable. The anti-V3 loop antibody titers of the vPE16 group were no greater than background after three immunizations with 5×10^6 pfu of recombinant vaccinia, and the lymphoproliferation responses of this group were also undetectable after two immunizations with a higher dose (1×10^6 pfu) of recombinant vaccinia. The vTA160/L1 vaccination group showed a lymphoproliferation response that was approximately 7 times greater than the vPE16 group at the maximum response level of the assay. The antibody response was also markedly enhanced; at a dilution of 1:1000, serum from mice vaccinated with vTA160/L1 showed an increasing amount of V3 loop antibodies during the course of the experiment. This relatively high titer antibody response, despite the targeting of the protein to lysosomes and the ensuing rapid degradation, suggests that some of the protein escaped to the extracellular milieu to interact with B cells, possibly by gp120 subunit dissociation from the gp41/LAMP component and subsequent secretion, or the presence of a fraction of the gp160/LAMP protein at the cell surface. It is known that a small fraction of the native LAMP proteins are expressed on the plasma membrane (53, 54).

There have been several studies that have shown the generation of anti-gp160 immune responses in mice that had been vaccinated with recombinant vaccinia expressing the HIV-1 envelope protein (10, 55, 56). These studies utilized a variety of immunization protocols, some of which included boosting with recombinant protein. The difference in protocols between those reports and our studies precludes direct comparison. In general, one difference is the amount of recombinant vaccinia inoculated. We have initiated a quantitative dose-response analysis of vaccinated mice comparing the LAMP-targeted *versus* wild type gp160.

Our model for the enhanced immune response mediated by LAMP targeting of endogenous antigen proposes that a limiting element in the immune response is the quantity of antigen processed and presented in MHC II molecules by APC. The LAMP targeting of antigen can be considered to act as a $CD4^+$ T cell-specific molecular adjuvant, enhancing the activation of $CD4^+$ T cells and increasing the population of helper T cells responsive to a specific viral or tumor antigen. A recent investigation of the relationship between the number of T cell receptors triggered and T cell activation suggests a possible mechanism by which LAMP targeting of antigen enhances the $CD4^+$ T cell response. That study showed that T cells became activated, irrespective of the ligand, when a threshold of approximately 8000 T cell receptors (TCR) were triggered, and a reduction in the number of the TCR expressed severely compromised the capacity of the T cell to reach the activation threshold. Cells with a low number of TCR required up to 1000 times greater concentration of antigen for activation (57). These results support the model that an increase in antigenic peptide-loaded MHC II resulting from LAMP-targeted antigen may more efficiently reach the TCR triggering threshold and activate T cells that express too few TCR to be effectively triggered by an equivalent concentration of non-targeted antigen.

Several studies have recently shown that MHC II molecules co-localize with LAMP in a unique multilaminar compartment, termed MIIC by some (32–35). This compartment appears to be another vesicular compartment accessed by the LAMP molecules or an intermediate compartment in the pathway of LAMP trafficking to lysosomes, and is potentially the site of antigen processing and peptide loading onto MHC II molecules (31–35, 58). Our results of an enhanced $CD4^+$ T cell proliferation stimulated by APC transfected with the gene encoding a LAMP-targeted antigen as compared with wild type antigen is consistent with the presence of a processing compartment containing both MHC II and LAMP proteins. However, the site of antigen processing and peptide loading onto MHC II molecules is controversial, and some studies have reported that antigen processing and peptide loading onto MHC II molecules occur in a compartment that is largely devoid of LAMP molecules (59, 60). It should be noted that our results could theoretically result without the co-localization of MHC II and LAMP molecules, as the data do not rule out the possibility that proteolytic processing of antigen occurs in a LAMP-related compartment, that is devoid of MHC II, but facilitates peptide loading of the MHC II molecule in another compartment.

The LAMP targeting system has been studied with several antigens. Extensive *in vitro* studies of the influenza virus hemagglutinin (HA) protein have shown an increase in the lymphoproliferative responses to the LAMP-targeted HA, both of a single epitope included in a recombinant LAMP-1 construct and of the HA protein containing the LAMP transmembrane and cytoplasmic domains.[2] In another study, the indirect targeting of soluble gp120 to lysosomes by a CD4/LAMP chimera resulted in enhanced MHC II presentation of gp120 peptides as demonstrated by an increase in both the cytolytic and proliferative responses of the gp120-specific CD4+ T cells (38). Further data have come from the HPV-16 E7 studies. Mice that received the LAMP-targeted E7 vaccine had higher titers of anti-E7 antibodies and greater CTL and lymphoproliferative responses to E7 peptides (36). Other studies with this system demonstrated that 80% of mice vaccinated with the recombinant vaccinia expressing E7/LAMP then challenged with tumor, remained tumor-free after 3 months, whereas 100% mice receiving the vaccinia expressing wild type E7 developed tumors within 2 weeks of challenge. Vaccination with E7/LAMP was also shown to result in the cure of mice with small established tumors. *In vivo* antibody depletion of lymphocyte subsets showed that this tumor rejection required both $CD4^+$ and $CD8^+$ T cells (37).

This procedure for antigen targeting to the MHC II antigen processing pathway by the LAMP system may have human applications. Foremost is the use of LAMP targeting with DNA vaccines to enhance helper T cell response. This novel process acting on an endogenously synthesized protein is in contrast to the normal endocytic pathway for MHC II processing and presentation of foreign or non-endogenous antigens. The LAMP strategy would have the greatest impact in the case of DNA immunization, where the endogenously synthesized antigens have limited access to the MHC II processing and presentation pathway.

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REFERENCES

1. Cooney, E. L., McElrath, M. J., Corey, L., Hu, S., Collier, A. C., Arditti, D., Hoffman, M., Coombs, R. W., Smith, G. E., and Greenberg, P. D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1882–1886
2. Dolin, R. (1995) *J. Infect. Dis.* **172**, 1175–1183
3. el-Daher, N., Keefer, M. C., Reichman, R. C., Dolin, R., and Roberts, N. J., Jr. (1993) *J. Infect. Dis.* **168**, 306–313
4. Graham, B. S., Belshe, R. B., Clements, M. L., Dolin, R., Corey, L., Wright, P. F., Gorse, G. J., Midthun, K., Keefer, M. C., Roberts, N. J., Jr., Schwartz, D. H., Agosti, J. M., Fernie, B. F., Stablein, D. M., Montefiori, D. C., Lambert, J. S., Hu, S., Esterlitz, J. R., Lawrence, D. N., Koff, W. C., and the AIDS Vaccine Clinical Trials Network (1992) *J. Infect. Dis.* **166**, 244–252
5. Graham, B. S., Matthews, T. J., Belshe, R. B., Clements, M. L., Dolin, R., Wright, P. F., Gorse, G. J., Schwartz, D. H., Keefer, M. C., Bolognesi, D. P., Corey, L., Stablein, D. M., Esterlitz, J. R., Hu, S., Smith, G. E., Fast, P. E., Koff, W. C., and the NIAID AIDS Vaccine Clinical Trials Network (1993) *J. Infect. Dis.* **167**, 533–537

6. Graham, B. S., Gorse, G. J., Schwartz, D. H., Keefer, M. C., McElrath, M. J., Matthews, T. J., Wright, P. F., Belshe, R. B., Clements, M. L., Dolin, R., Corey, L., Bolognesi, D. P., Stablein, D. M., Esterlitz, J. R., Hu, S., Smith, G. E., and the National Institute of Allergy and Infectious Diseases AIDS Vaccine Clinical Trials Network (1994) *J. Infect. Dis.* **170**, 782–786
7. McElrath, M. J., Corey, L., Berger, D., Hoffman, M. C., Klucking, S., Dragavon, J., Peterson, E., and Greenberg, P. D. (1994) *J. Infect. Dis.* **169**, 41–47
8. Montefiori, D. C., Graham, B. S., Kliks, S., and Wright, P. F. (1992) *J. Clin. Immunol.* **12**, 429–439
9. Natuk, R. J., Lubeck, M. D., Chanda, P. K., Chengalvala, M., Wade, M. S., Murthy, S. C., Wilhelm, J., Vernon, S. K., Dheer, S. K., Mizutani, S., Lee, S., Murthy, K. K., Eichberg, J. W., Davis, A. R., and Hung, P. P. (1993) *AIDS Res. Hum. Retroviruses* **9**, 395–404
10. Parry, C., McLain, L., and Dimmock, N. J. (1994) *AIDS Res. Hum. Retroviruses* **10**, 205–212
11. Perales, M. A., Schwartz, D. H., Fabry, J. A., and Lieberman, J. (1995) *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* **10**, 27–35
12. Wang, B., Boyer, J., Srikantan, V., Ugen, K., Gilbert, L., Phan, C., Dang, K., Merva, M., Agadjanyan, M. G., Newman, M., Carrono, R., McCallus, D., Coney, L., Williams, W. V., and Weiner, D. B. (1995) *Virology* **211**, 102–112
13. Wang, B., Merva, M., Dang, K., Ugen, K. E., Boyer, J., Williams, W. V., and Weiner, D. B. (1994) *AIDS Res. Hum. Retroviruses* **10**, Suppl. 2, S35–S41
14. Wang, B., Boyer, J., Srikantan, V., Coney, L., Carrano, R., Phan, C., Merva, M., Dang, K., Agadjanan, M., Gilbert, L., Ugen, K. E., Williams, W. V., and Weiner, D. B. (1993) *DNA Cell Biol.* **12**, 799–805
15. Lu, S., Santoro, J. C., Fuller, D. H., Haynes, J. R., and Robinson, H. L. (1995) *Virology* **209**, 147–154
16. Haynes, J. R., Fuller, D. H., Eisenbraun, M. D., Ford, M. J., and Pertmer, T. M. (1994) *AIDS Res. Hum. Retroviruses* **10**, Suppl. 2, S43–S45
17. Fuller, D. H., and Haynes, J. R. (1994) *AIDS Res. Hum. Retroviruses* **10**, 1433–1441
18. Abbas, A. K., Lichtman, A. H., and Pober, J. S. (1994) *Cellular and Molecular Immunology*, 2nd Ed., pp. 116–135, W. B. Saunders, Philadelphia
19. Cresswell, P. (1994) *Annu. Rev. Immunol.* **12**, 259–293
20. Yewdell, J. W., and Bennink, J. R. (1990) *Cell* **62**, 203–206
21. Rowell, J. F., Stanhope, P. E., and Siliciano, R. F. (1995) *J. Immunol.* **155**, 473–488
22. Polydefkis, M., Koenig, S., Flexner, C., Obah, E., Gebo, K., Chakrabarti, S., Earl, P. L., Moss, B., and Siliciano, R. F. (1990) *J. Exp. Med.* **171**, 875–887
23. Eager, K. B., Hackett, C. J., Gerhard, W. U., Bennink, J., Eisenlohr, L. C., Yewdell, J., and Ricciardi, R. P. (1989) *J. Immunol.* **143**, 2328–2335
24. Calin-Laurens, V., Forquet, F., Lombard-Platet, S., Bertolino, P., Chretien, I., Trescol-Biemont, M. C., Gerlier, D., and Rabourdin-Combe, C. (1992) *Int. Immunol.* **4**, 1113–1121
25. Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I., and August, J. T. (1985) *J. Cell Biol.* **101**, 85–95
26. Chen, J. W., Cha, Y., Yuksel, K. U., Gracy, R. W., and August, J. T. (1988) *J. Biol. Chem.* **263**, 8754–8758
27. Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y., and August, J. T. (1993) *J. Biol. Chem.* **268**, 1941–1946
28. Castellino, F. (1995) *Immunity* **2**, 73–88
29. Harding, C. V., and Geuze, H. J. (1992) *J. Cell. Biol.* **119**, 531–542
30. Harding, C. V., and Geuze, H. J. (1993) *J. Immunol.* **151**, 3988–3998
31. Qiu, Y., Xu, X., Wandinger-Ness, A., Dalke, D. P., and Pierce, S. K. (1994) *J. Cell Biol.* **125**, 595–605
32. Tulp, A., Verwoerd, D., Dobberstein, B., Ploegh, H. L., and Pieters, J. (1994) *Nature* **369**, 120–126
33. Peters, P. J., Raposo, G., Neefjes, J. J., Oorschot, V., Leijendekker, R. L., Geuze, H. J., and Ploegh, H. L. (1995) *J. Exp. Med.* **182**, 325–334
34. Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L., and Geuze, H. J. (1991) *Nature* **349**, 669–676
35. Kleijmeer, M. J., Ossevoort, M. A., van Veen, C. J., van Hellemond, J. J., Neefjes, J. J., Kast, W. M., Melief, C. J., and Geuze, H. J. (1995) *J. Immunol.* **154**, 5715–5724
36. Wu, T. C., Guarnieri, F. G., Staveley-O'Carroll, K. F., Viscidi, R. P., Levitsky, H. I., Hedrick, L., Cho, K. R., August, J. T., and Pardoll, D. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11671–11675
37. Lin, K., Guarnieri, F. G., Staveley-O'Carroll, K. F., Levitsky, H. I., August, J. T., Pardoll, D. M., and Wu, T. C. (1996) *Cancer Res.* **56**, 21–26
38. Rowell, J. F., Ruff, A. L., Guarnieri, F. G., Staveley-O'Carroll, K., Lin, X., Tang, J., August, J. T., and Siliciano, R. F. (1995) *J. Immunol.* **155**, 1818–1828
39. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, J. S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., and Wong-Stall, F. (1985) *Nature* **313**, 277–284
40. Earl, P. L., Hugin, A. W., and Moss, B. (1990) *J. Virol.* **64**, 2448–2451
41. Earl, P., Koenig, S., and Moss, B. (1991) *J. Virol.* **65**, 31–41
42. Moss, B., and Earl, P. E. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K., eds) Vol. 2, pp. 16.15.1–16.15.5, John Wiley & Sons, New York
43. Moss, B., Earl, P. E., and Cooper, N. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K., eds) Vol. 2, pp. 16.16.1–16.16.7, John Wiley & Sons, New York
44. Moss, B., and Earl, P. E. (1993) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K., eds) Vol. 2, pp. 16.17.1–16.17.16, John Wiley & Sons, New York
45. Pincus, S. H., Wehrly, K., and Chesebro, B. (1989) *J. Immunol.* **142**, 3070–3075
46. Siliciano, R. F., Lawton, T., Knall, C., Karr, R. W., Berman, P., Gregory, T., and Reinherz, E. L. (1988) *Cell* **54**, 561–575
47. Orentas, R. J., Hildreth, J. E. K., Obah, E., Polydefkis, M., Smith, G. E., Clements, M. L., and Siliciano, R. F. (1990) *Science* **248**, 1234–1237
48. Hammond, S. A., Bollinger, R. C., Stanhope, P. E., Quinn, T. C., Schwartz, D., Clements, M. L., and Siliciano, R. F. (1992) *J. Exp. Med.* **176**, 1531–1542
49. Hathcock, K. S. (1991) in *Current Protocols in Immunology* (Cloigan, J. E., Kruisbeek, A. M., Marguiles, D. H., Shevach, E. M., and Strober, W., eds) Vol. 1, pp. 3.2.1–3.2.4, John Wiley & Sons, New York
50. Hale, P. M., Cease, K. B., Houghten, R. A., Ouyang, C., Putney, S., Javaherian, K., Margalit, H., Cornette, J. L., Spouge, J. L., DeLisi, C., and Berzofsky, J. A. (1989) *Int. Immunol.* **1**, 409–415
51. Willey, R. L., Bonifacino, J. S., Potts, B. J., Martin, M. A., and Klausner, R. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 9580–9584
52. D'Souza, M. P., and August, J. T. (1986) *Arch. Biochem. Biophys.* **249**, 522–532
53. Mathews, P. M., Martinie, J. B., and Fambrough, D. M. (1992) *J. Cell Biol.* **118**, 1027–1040
54. Hughes, E. N., and August, J. T. (1981) *J. Biol. Chem.* **256**, 664–671
55. Michel, F., Hoffenbach, A., Langlade-Demoyen, P., Guy, B., Girard, M., Lecocq, J. P., Wain-Hobson, S., Kieny, M. P., and Plata, F. (1988) *Eur. J. Immunol.* **18**, 1917–1924
56. Hu, S. L., Klaniecki, J., Dykers, T., Sridhar, P., and Travis, B. M. (1991) *AIDS Res. Hum. Retroviruses* **7**, 615–620
57. Viola, A., and Lanzavecchia, A. (1996) *Science* **273**, 104–106
58. Rudensky, A. Y., Maric, M., Eastman, S., Shoemaker, L., DeRoos, P. C., and Blum, J. S. (1994) *Immunity* **1**, 585–94
59. Amigorena, S., Drake, J. R., Webster, P., and Mellman, I. (1994) *Nature* **369**, 113–120
60. Arkema, J. M., Schadee-Eestermans, I. L., Broekhuis-Fluitsma, D. M., Fukuda, M., and Hoefsmit, E. C. (1994) *Histochem. J.* **26**, 95–99

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Immunology

Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens

(human papillomavirus/LAMP-1/recombinant vaccinia)

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ABSTRACT **The presentation of antigenic peptides by major histocompatibility complex (MHC) class II molecules to CD4+ T cells is critical to the function of the immune system. In this study, we have utilized the sorting signal of the lysosomal-associated membrane protein LAMP-1 to target a model antigen, human papillomavirus 16 E7 (HPV-16 E7), into the endosomal and lysosomal compartments. The LAMP-1 sorting signal reroutes the antigen into the MHC class II processing pathway, resulting in enhanced presentation to CD4+ cells *in vitro*. *In vivo* immunization experiments in mice demonstrated that vaccinia containing the chimeric E7/LAMP-1 gene generated greater E7-specific lymphoproliferative activity, antibody titers, and cytotoxic T-lymphocyte activities than vaccinia containing the wild-type HPV-16 E7 gene. These results suggest that specific targeting of an antigen to the endosomal and lysosomal compartments enhances MHC class II presentation and vaccine potency.**

The presentation of antigenic peptides by major histocompatibility complex (MHC) class II molecules to CD4+ T cells is critical to the function of the immune system. CD4+ T cells are the major helper T-cell phenotype whose predominant function is to generate cytokines that regulate essentially all other functions of the immune response. CD4+ MHC class II restricted cells have also been shown to have cytotoxic capacity in a number of systems, including a response to fragments of the human immunodeficiency virus gp120 protein (1). CD4+ cells have also been shown to be of great importance in immune responses against several different murine (2, 3) and human (4) solid malignancies. Several mouse tumors that were transfected with syngeneic MHC class II genes have become very effective vaccines against subsequent challenge with wild-type (wt) class II negative tumors (5). For these reasons, there has been increased interest in developing strategies that will most effectively activate CD4+ MHC class II restricted cells against a given specific antigen (6).

Two major pathways by which antigens enter endosomal and lysosomal compartments for MHC class II presentation to CD4+ T cells have been described. The traditional pathway involves the phagocytosis or endocytosis of exogenous proteins into antigen-presenting cells (APCs), followed by degradation by acid proteases in the endosomal or lysosome-like compartments (7–9). A second pathway involves the processing of membrane proteins endogenously synthesized by APCs (1, 10). These membrane proteins are believed to enter endosomal and lysosomal compartments by internalization from the cell surface. In certain experimental systems, cytoplasmic proteins may also enter this endogenous MHC class II pathway (11, 12), but normally these antigens are preferentially routed for MHC class I presentation. In general, cytoplasmic or nuclear proteins are degraded into peptides in the cytoplasm, which are then transported into the endoplasmic reticulum where they complex with newly assembled class I molecules on their way to the cell surface for presentation to CD8+ T cells (for review, see ref. 13).

We reasoned that a molecular approach that directly routes an antigen into the MHC class II processing and presentation pathway, such as endosomal and lysosomal compartments, might enhance its presentation to MHC class II restricted CD4+ T cells. The endosomal and lysosomal compartments are characterized by the presence of several compartment-specific membrane proteins. The lysosomal-associated membrane protein LAMP-1 is a type 1 transmembrane protein localized predominantly to lysosomes and late endosomes (14, 15). The cytoplasmic domain of LAMP-1 contains the amino acid sequence Tyr-Gln-Thr-Ile, whose structure conforms to Tyr-Xaa-Xaa-hydrophobic amino acid motif that mediates cell membrane internalization and possibly lysosomal targeting of several cell surface receptors (16–20). The intracellular targeting of LAMP-1 has been shown to be controlled by the amino acid sequence Tyr-Gln-Thr-Ile at the C terminus of its cytoplasmic tail (21, 22). We therefore engineered a chimeric gene encoding a model antigen linked to the transmembrane and cytoplasmic region of LAMP-1, which we hypothesized would target the antigen to the endosomal and lysosomal compartments.

We chose the human papillomavirus 16 E7 (HPV-16 E7) as a model antigen for two reasons: (*i*) HPV-16 E7 is a characterized cytoplasmic/nuclear protein (23–26) and (*ii*) immunological studies targeting HPV-16 E7 can potentially lead to development of therapeutic vaccines against HPV-associated malignancies (for reviews, see ref. 27). In this study, our data suggest that specific targeting of HPV-16 E7 to the endosomal and lysosomal compartments enhances MHC class II presentation and results in increased vaccine potency *in vivo*.

MATERIALS AND METHODS

DNA Constructs. For generation of the Sig/E7/LAMP-1 chimeric gene, DNA fragments encoding the signal peptide (N-terminal) of LAMP-1, the open reading frame of HPV-16 E7, and the transmembrane domain and cytoplasmic tail of LAMP-1 were amplified by PCR using high-fidelity *Pfu* polymerase (Stratagene). The primer sets were designed so that *Bgl* II and *Bam*HI restriction sites were generated at the 5′ and 3′ ends of the amplified fragments, respectively. The primer set for signal peptide of LAMP-1 was 5′-TTGAGATCTTATG-

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Abbreviations: MHC, major histocompatibility complex; wt, wild-type; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; LAMP-1, lysosomal-associated membrane protein; mAb, monoclonal antibody; HPV, human papillomavirus; pfu, plaque-forming units.

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GCGGCCCCC-3′ and 5′-TTGGGATCCTCAAAGAGTGCTGA-3′. The primer set for HPV-16 E7 open reading frame was 5′-CCCAGATCTAATCATGCATG-3′ and 5′-TATGGATCCTGAGAACAGAT-3′. The primer set for the transmembrane and cytoplasmic domain of LAMP-1 was 5′-TCAAGATCTTAACAACATGTTG-3′ and 5′-TGTGGATCCCTTCCACACC-3′. The amplified DNA fragments were cloned sequentially into the unique *Bam*HI cloning site of the pCMVneo expression vector (28) downstream of the cytomegalovirus promoter.

Transfections and Immunofluorescence Stainings. Transfections and immunofluorescent stainings were performed as described (22). Mouse anti-HPV-16 E7 monoclonal antibody (mAb) (Triton, San Diego) was used to detect HPV-16 E7. Rat anti-mouse LAMP-1 mAb (1D4B) (14) was used to detect LAMP-1.

Proliferation Assay. Proliferation assays were performed following the protocol of Kruisbeek and Shevach (29). For *in vitro* assays, CBF1 mice were injected in the foot pad with 20 μg of HPV-16 E7 peptide (aa 30–67) mixed with complete Freund's adjuvant. Lymphocytes were prepared by crushing peripheral lymph nodes from CBF1 mice 2 wk after *in vivo* priming. An aliquot of 3×10^5 cells was plated in triplicate in 96-well plates with decreasing dilutions of irradiated (5000 rad; 1 rad = 0.01 Gy) LB27 APCs stably transfected with E7, Sig/E7, or Sig/E7/LAMP-1. For *in vivo* assays, lymphocytes from peripheral lymph nodes of vaccinia-infected C57BL/6 mice or CD4-knockout C57BL/6 mice were mixed with decreasing dilutions of E7 30–67 peptides.

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses were generated following the protocol of Earl and Moss (30–32). Plaque-purified recombinant vaccinia viruses were tested for the presence of HPV-16 E7 genome by PCR and for the expression of HPV 16 E7 protein by immunofluorescent stainings.

Cytotoxic T-Lymphocyte (CTL) Assays. Induction and measurement of CTL activity were performed using standard protocols (33). Splenocytes from BALB/c, C57BL/6, or CD4-knockout C57BL/6 mice were used for CTL assays. Splenocytes were harvested 2 wk after mice were infected with 10^7 plaque-forming units (pfu) of recombinant vaccinia by i.p. injection. Splenocytes were cocultured with mitomycin C-treated HPV-16 E7 containing syngeneic tumor cells (stimulators) for 6 days. CTL assays were performed in a standard 4-hr chromium release assay. Target cell-specific lysis was determined by subtracting each sample's % of wt target cell CD8-blockable lysis from its corresponding % of E7 target cell CD8-blockable lysis.

ELISA. The anti-HPV-16 E7 antibodies in the sera from recombinant vaccinia-infected C57BL/6 mice or CD4-knockout C57BL/6 mice (34) were determined by ELISA using microwell plates coated with synthetic E7 peptides (aa 30–67) or yeast-derived HPV-16 E7 protein prepared as described (35).

RESULTS

Generation of Chimeric Sig/E7/LAMP-1 Molecule. To target HPV-16 E7, a cytoplasmic and nuclear protein, to the endosomal and lysosomal compartments, it was first necessary to place a signal peptide at the N terminus of the protein to mediate translocation into the lumen of the endoplasmic reticulum. The transmembrane domain and cytoplasmic tail of LAMP-1 were placed at the C terminus of the E7 protein because these components are known to confer endosomal/lysosomal targeting (22). The DNA fragments that encode the N-terminal signal peptide sequence of LAMP-1, HPV-16 E7, and the transmembrane and cytoplasmic domains of LAMP-1 were amplified by PCR and sequentially cloned into a mammalian expression vector. Fig. 1 shows a diagram of the final construct, which was confirmed by DNA sequencing. The chimeric Sig/E7 is the intermediate product of Sig/E7/LAMP-1. Sig/E7 was generated by placing the signal peptide of LAMP-1 at the N terminus of the HPV-16 E7.

Addition of LAMP-1 Signal Peptide, Transmembrane, and Cytoplasmic Portions onto HPV-16 E7 Protein Efficiently Reroutes E7 into the Endosomal/Lysosomal Compartment. Transfections and subsequent immunofluorescent stainings were used to determine expression and localization of wt and modified HPV-16 E7 protein. As expected, cells transfected with wt E7 showed homogeneous cytoplasmic/nuclear staining (Fig. 2*B*). In comparison, cells transfected with the chimeric Sig/E7/LAMP-1 construct displayed a vesicular pattern consistent with endosomal and lysosomal localization (Fig. 2*A*). To further confirm localization of the Sig/E7/LAMP-1 chimera to the endosomal and lysosomal compartments, we performed double labeling experiments of cells stably transfected with Sig/E7/LAMP-1 using antibodies against HPV-16 E7 and the N-terminal (or luminal) portion of endogenous LAMP-1, a well-characterized marker for the endosomal and lysosomal compartments. Colocalization of E7 and endogenous LAMP-1 was clearly visible (Fig. 3), indicating that the Sig/E7/LAMP-1 chimera was indeed targeted to the endosomal and lysosomal compartments (see Fig. 3 *Insets*). Controls omitting primary antibodies did not show specific staining (data not shown).

Modification of Endogenously Synthesized E7 with the LAMP-1 Sorting Signal Enhances Presentation to MHC II Restricted T Cells. To demonstrate that the Sig/E7/LAMP-1 chimeric protein efficiently enters the MHC class II processing

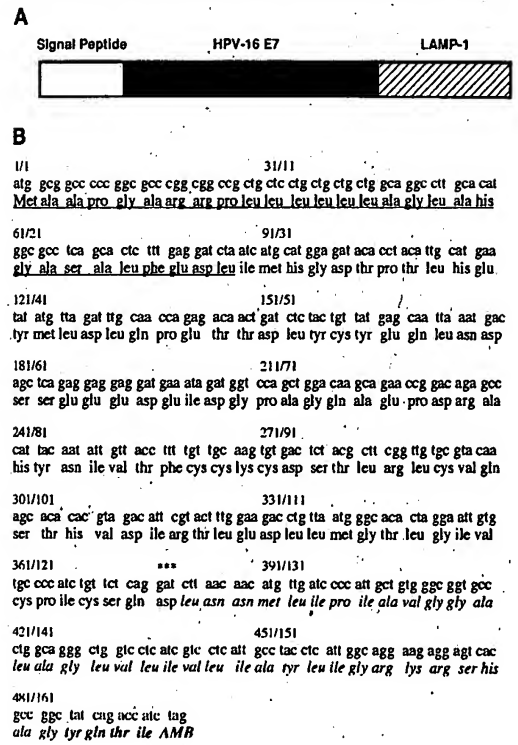

FIG. 1. Diagram and nucleotide sequence of the chimeric Sig/E7/LAMP-1 protein. (*A*) Diagram of chimeric Sig/E7/LAMP-1. The signal peptide is white, HPV-16 E7 is black, and the LAMP-1 transmembrane and cytoplasmic tail is hatched. (*B*) Nucleotide sequence of chimeric Sig/E7/LAMP-1. The signal peptide is underlined. The LAMP-1 transmembrane and cytoplasmic tail is in italics. The lysosomal targeting signal is in boldface. Asterisks (***) indicate the last amino acid of chimeric Sig/E7 protein (127 aa).

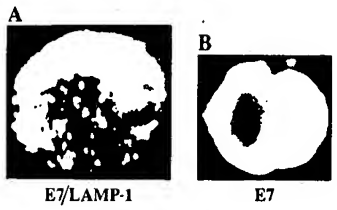

FIG. 2. Immunofluorescent staining of HPV-16 E7 and E7/LAMP-1 chimeric proteins in the transfected cells. Human embryonic kidney cells, 293S, were transiently transfected with pCMVneo/Sig/E7/LAMP-1 (*A*) or pCMVneo-16E7 (*B*) by calcium phosphate-DNA coprecipitation. 293S cells were fixed, permeabilized, and stained with mouse anti-HPV-16 E7 mAb followed by Texas red-conjugated goat anti-mouse secondary antibody (*A* and *B*). Note the positive vesicular patterns that were appreciated in cells transfected with Sig/E7/LAMP-1 recombinant plasmid (*A*). In contrast, diffuse cytoplasmic as well as nuclear stainings were noted in cells transfected with wt HPV-16 E7 plasmid (*B*). (×500.)

and presentation pathway, we assayed for stimulation of E7-specific MHC class II restricted proliferative responses. LB27, a B-cell hybridoma expressing I-$A^{b,d}$ and I-$E^{b,d}$ MHC class II molecules as APCs, was transfected with either wt HPV-16 E7, Sig/E7, or Sig/E7/LAMP-1 genes in plasmids encoding neomycin resistance. G418-resistant clones were isolated by limiting dilution and tested for the presence of HPV-16 E7 or Sig/E7/LAMP-1 by PCR and immunofluorescent stainings (data not shown). HPV-16 E7-specific lymphocytes were generated by injecting the foot pad of CBF1 mice with HPV-16 E7 peptide (aa 30–67) emulsified in complete Freund's adjuvant. This HPV-16 E7 peptide has been reported to contain a T-helper cell epitope (aa 44–60) capable of being presented by multiple MHC class II alleles (36). The HPV-16 E7 peptide-primed lymphocytes showed specific proliferative responses to E7 44–60 and E7 30–67 (data not shown). Fig. 4 compares the ability of E7, Sig/E7, and Sig/E7/LAMP-1 transfected LB27 cells to stimulate E7 30–67-specific T cells. E7 and Sig/E7 transfectants fail to stimulate E7 30–67-specific T cells, consistent with the inefficient entry of intracellular proteins into the MHC class II processing pathway. In contrast, Sig/E7/LAMP-1 expressing LB27 cells efficiently stimulated HPV-16 E7 30–67-specific T cells. These results demonstrate that the Sig/E7/LAMP-1 chimeric protein is efficiently processed and presented in the MHC class II pathway. Virtually identical results were obtained when responder T cells were depleted of $CD8^+$ cells, demonstrating that the enhanced proliferative response to Sig/E7/LAMP-1 transfectants is predominantly due to $CD4^+$ activation (data not shown).

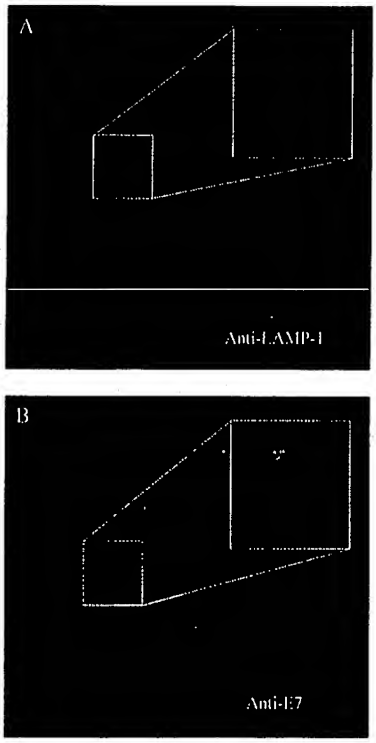

FIG. 3. Colocalization of E7 and LAMP-1 proteins in cell transfected with chimeric Sig/E7/LAMP-1 by double labeling immunofluorescent stainings. LB27 cells were stably transfected with pCMVneo/Sig/E7/LAMP-1. Double labeling immunofluorescent stainings were performed by first incubating transfected LB27 cells with the mouse anti-HPV-16 E7 mAb and the rat anti-mouse LAMP-1 mAb (1D4B) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and Texas red-conjugated goat anti-rat IgG secondary antibodies. For detection of endogenous LAMP-1, Texas red fluorescent staining was noted (*A*). For detection of HPV-16 E7 protein, FITC fluorescent staining was noted (*B*). (*Insets*) Enlargements of the same compartments that were positive for E7 protein and endogenous LAMP-1. Controls that omitted primary antibodies did not show specific staining (data not shown). (×125.)

Modification of E7 with the LAMP-1 Sorting Signal Enhances *in Vivo* Immune Responses Induced by Recombinant Viral Vaccines. To measure the effect of LAMP-1 targeting of antigen *in vivo*, we selected the vaccinia virus system. Vaccinia virus has a large capacity for genetic insertion (37). Since vaccinia immunization has been postulated to involve direct infection of APCs, it represented a good vaccine system to analyze the consequences of LAMP-1-mediated targeting of antigen *in vivo*. Plaque-purified recombinant vaccinia viruses were tested for the presence of the HPV-16 E7 and Sig/E7/LAMP-1 genes by PCR. Immunofluorescence was used to determine expression and localization of HPV-16 E7 and Sig/E7/LAMP-1 in recombinant vaccinia-infected cells. The pattern of staining of cells infected with recombinant vaccinia expressing HPV-16 E7 or Sig/E7/LAMP was similar to that

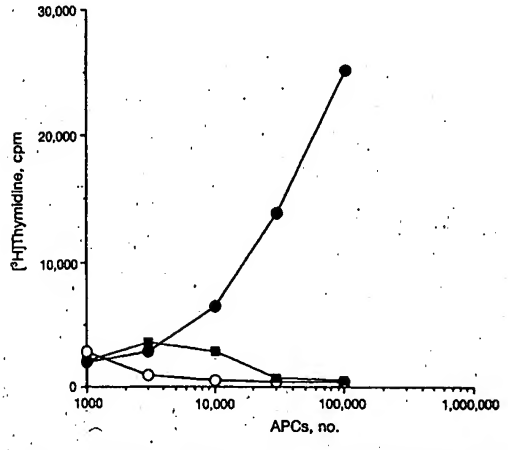

FIG. 4. Proliferation of HPV-16 E7 peptide-primed lymphocytes in response to LB27 APCs transfected with HPV-16 E7, Sig/E7, or Sig/E7/LAMP-1. LB27 cells transfected with Sig/E7/LAMP-1 (solid circles), HPV-16 E7 (open circles), or Sig/E7 (solid squares) were irradiated and coincubated with HPV-16 E7 peptide-specific T cells derived from peripheral draining lymph nodes of CBF1 mice primed with HPV-16 E7 peptide (aa 30–67) in complete Freund's adjuvant. T-cell proliferation was measured by incorporation of [^{3}H]thymidine.

observed with transfection of recombinant plasmids as described above (data not shown), indicating that the vesicular localization of Sig/E7/LAMP-1 product was independent of the expression vector.

We first measured the generation of E7-specific proliferative responses as a measure of T-helper cell activation. Significantly greater lymphoproliferative activity was observed with T cells from C57BL/6 mice infected with Sig/E7/LAMP-1 vaccinia than with wt HPV-16 E7 vaccinia (Fig. 5). The proliferation was HPV-16 E7 peptide specific, since no specific proliferation was observed when pulsed with control peptide (data not shown). Furthermore, CD4-knockout mice did not show significant lymphoproliferative activity compared to that of C57BL/6 mice. These results demonstrated that the endosomal and lysosomal targeting of HPV-16 E7 enhanced presentation of E7 peptides to $CD4^+$ T-helper cells.

To measure anti-HPV-16 E7 antibody production, we performed ELISA using plates coated with synthetic HPV-16 E7 peptides or yeast-derived HPV-16 E7 protein (35). Again, the highest titers of anti-HPV-16 E7 antibodies were detected in sera from C57BL/6 mice primed with the chimeric Sig/E7/LAMP-1 expressing vaccinia (Fig. 6). In comparison, anti-HPV-16 E7 antibodies were significantly decreased in sera from CD4-knockout C57BL/6 mice infected with chimeric Sig/E7/LAMP-1 expressing vaccinia (Fig. 6). Finally, we analyzed vaccinia E7 and vaccinia Sig/E7/LAMP-1 immunized mice for E7-specific CTL activity. It was certainly possible that the LAMP-1-mediated targeting of E7 into the MHC class II processing pathway might interfere with processing of MHC class I epitopes. This was not found to be the case since the highest CTL activities were noted in mice that were primed with Sig/E7/LAMP-1 vaccinia (Fig. 7*A*). Such results were observed with C57BL/6 and BALB/c mice (BALB/c data not shown). The enhanced CTL activity generated by the chimeric Sig/E7/LAMP-1 vaccinia was most likely due to an increase in $CD4^+$ T-cell help via improved presentation of MHC class II restricted epitopes, since significant loss of CTL activity was observed in CD4-knockout C57BL/6 mice (Fig. 7*B*).

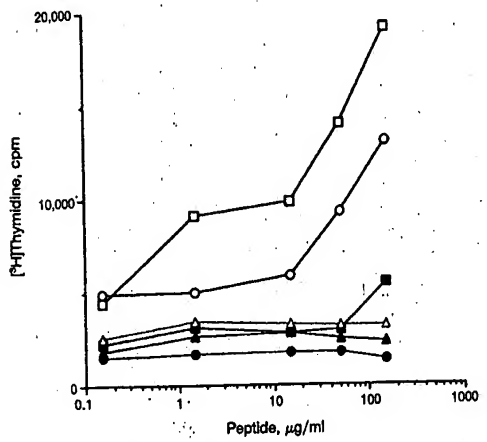

FIG. 5. E7-specific proliferative response of lymphocytes from mice immunized with various recombinant vaccinia. Lymphocytes were prepared from C57BL/6 (open symbols) or CD4-knockout C57BL/6 (solid symbols) mice immunized with 10^7 pfu of Sig/E7/LAMP-1 (squares), HPV-16 E7 (circles), or wt (triangles) recombinant vaccinia virus. Two weeks after infection, splenocytes were isolated and T cells were purified over a nylon wool column. T cells were mixed with naive C57BL/6 or CD4-knockout C57BL/6 splenocytes with decreasing dilutions of E7 30–67 peptide. Proliferation was measured by incorporation of [^{3}H]thymidine.

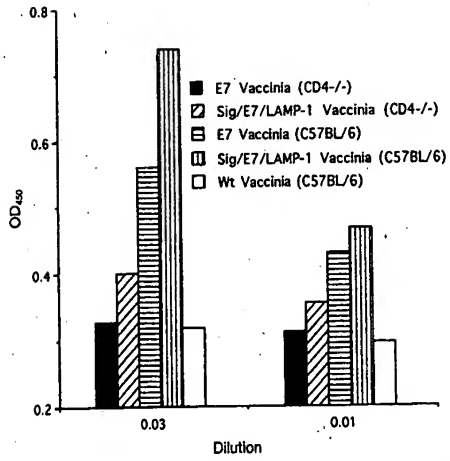

FIG. 6. E7-specific antibody response in C57BL/6 or CD4-knockout C57BL/6 mice immunized with various recombinant vaccinia. C57BL/6 were immunized with wt vaccinia, HPV-16 E7 vaccinia, or Sig/E7/LAMP-1 vaccinia at a dose of 10^7 pfu per mouse. Similarly, CD4-knockout C57BL/6 mice were immunized with HPV-16 E7 vaccinia or E7/LAMP-1 vaccinia at a dose of 10^7 pfu per mouse. Serum samples were obtained from mice 2 wk after infection. ELISA was then performed.

These results clearly demonstrate that the endosomal and lysosomal targeting of HPV-16 E7 enhanced the priming of MHC class II and MHC class I restricted T cells.

DISCUSSION

In this study we have utilized the targeting signal of the lysosomal membrane protein LAMP-1 to direct a model cytoplasmic/nuclear antigen, HPV-16 E7, into the endosomal and lysosomal compartments. This unique approach not only concentrates the antigen in these compartments but also leads to enhanced MHC class II presentation. Furthermore, *in vivo* data suggested that this strategy might enhance the priming of MHC class I restricted T cells as well.

Although our experiments do not directly address the localization of MHC class II peptide loading, the data certainly indicate that antigens directed through the LAMP-1 targeting pathway show enhanced MHC class II restricted $CD4^+$ T-cell stimulation. Antigen breakdown may be initiated within proteolytically active endosomal and lysosomal compartments (39–41) and there may exist additional intracellular trafficking pathways for the processed peptides to transfer from the endosomes and lysosomes to compartments where peptides bind with MHC class II molecules.

We also observed enhanced CTL activity in mice infected with E7/LAMP-1 recombinant vaccinia. The ability of LAMP-1-targeted E7 to generate increased CTL activity in mice appears paradoxical since MHC class I restricted epitopes in the wt HPV-16 E7 would be expected to have access to the highly efficient classical MHC class I processing and presentation pathway for nuclear and cytoplasmic antigens. On the other hand, peptides from membrane-associated proteins are also efficiently presented by MHC class I via the transporter associated with antigen processing (TAP)-dependent and TAP-independent pathways (42). Clearly, CTL epitopes from the chimeric Sig/E7/LAMP-1 product are presented on MHC class I molecules. We speculated that the enhanced CTL activity in C57BL/6 mice infected with the chimeric Sig/E7/LAMP-1 vaccinia might be due to an increase in $CD4^+$ T-cell help as a result of improved presentation of MHC class II restricted epitopes since similarly

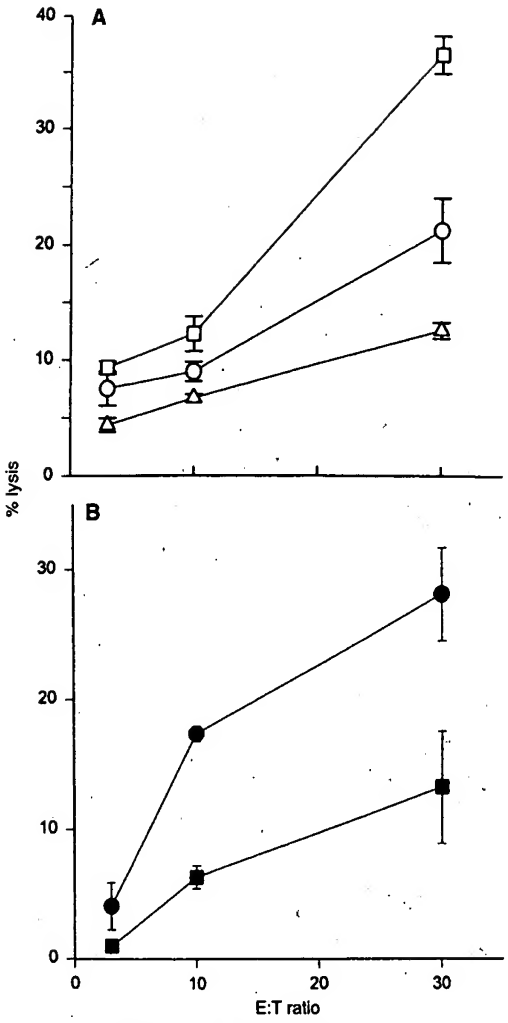

FIG. 7. E7-specific CTL response in C57BL/6 mice immunized with various recombinant vaccinia. (*A*) C57BL/6 mice were immunized i.p. with 1×10^7 pfu of wt vaccinia (open triangles), HPV-16 E7 vaccinia (open circles), or Sig/E7/LAMP-1 vaccinia (open squares). (*B*) C57BL/6 (solid circles) or CD4-knockout mice (solid squares) were immunized with 1×10^7 pfu of Sig/E7/LAMP-1 vaccinia. Splenocytes were harvested 2 wk after infection. Splenocytes were stimulated *in vitro* with mitomycin C-treated MC57G-E7, an H-2^b tumor cell, for 6 days before ^{51}Cr release assays were performed. MC57G-E7 or MC57G cells were used as target cells. CD8-blockable lysis was ascertained by incubating splenocytes with rat anti-CD8 mAb (2.43) (38) for 30 min before the ^{51}Cr release assay was performed. Target cell-specific lysis was determined by subtracting each sample's % of MC57G target cell CD8-blockable lysis from its corresponding % of MC57G-E7 cell CD8-blockable lysis. E:T, effector:target. Values are expressed as mean ± SEM.

treated CD4-knockout C57BL/6 mice significantly lost the enhanced CTL activity.

Our results have a number of implications for MHC class II-dependent antigen presentation and for vaccine design in general. They demonstrate that specific intracellular antigen-targeting strategies can be successfully utilized to enhance the presentation of antigenic epitopes, thereby increasing T-cell stimulation. For such a strategy to effectively enhance vaccine potency, the form of vaccination would require that expression of the LAMP-1-targeted antigen be endogenous in APCs. Our data with recombinant vaccinia are consistent with the notion that the induction of immune responses involves direct infection of APCs by vaccinia *in vivo*. An additional strategy for vaccine design employing enhanced MHC class II targeting might involve the direct introduction of LAMP-1-tagged antigens into autologous APCs *ex vivo* followed by their reinfusion.

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1. Polydefkis, M., Koenig, S., Flexner, C., Obah, E., Gebo, K., Chakrabarti, S., Earl, P. L., Moss, B. & Siliciano, R. F. (1990) *J. Exp. Med.* **171**, 875–887.
2. Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., Karasuyama, H., Vogelstein, B. & Frost, P. (1990) *Cell* **60**, 397–403.
3. Golumbek, P. T., Lazenby, A. J., Levitsky, H. I., Jaffee, L. M., Karasuyama, H., Baker, M. & Pardoll, D. M. (1991) *Science* **254**, 713–716.
4. Topalian, S. L., Rivoltini, L., Mancini, M., Markus, N. R., Robbins, P. F., Kawakami, Y. & Rosenberg, S. A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9461–9465.
5. Ostrand-Rosenberg, S., Thakur, A. & Clements, V. (1990) *J. Immunol.* **144**, 4068–4071.
6. Pardoll, D. M. (1993) *Curr. Opin. Immunol.* **5**, 719–725.
7. Blum, J. S. & Cresswell, P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3975–3979.
8. Neefjes, J. J., Stollorz, V., Peters, P. J., Geuze, H. J. & Ploegh, H. L. (1990) *Cell* **61**, 171–183.
9. Yewdell, J. W. & Bennink, J. R. (1990) *Cell* **62**, 203–206.
10. Eager, K. B., Hackett, C. J., Gerhard, W. U., Bennink, J., Eisenlohr, L. C., Yewdell, J. & Ricciardi, R. P. (1989) *J. Immunol.* **143**, 2328–2335.
11. Jaraquemada, D., Marti, M. & Long, E. O. (1990) *J. Exp. Med.* **172**, 947–954.
12. Nuchtern, J. G., Biddison, W. E. & Klausner, R. D. (1990) *Nature (London)* **343**, 74–76.
13. Germain, R. N. (1986) *Nature (London)* **322**, 687–689.
14. Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I. & August, J. T. (1985) *J. Cell Biol.* **101**, 85–95.
15. Lewis, V., Green, S. A., Marsh, M., Vihko, P., Helenius, A. & Mellman, I. (1985) *J. Cell Biol.* **100**, 1839–1847.
16. Breitfeld, P. P., Casanova, J. E., McKinnon, W. C. & Mostov, K. E. (1990) *J. Biol. Chem.* **265**, 13750–13757.
17. Chen, W. J., Goldstein, J. L. & Brown, M. S. (1990) *J. Biol. Chem.* **265**, 3116–3123.
18. Collawn, J. F., Lai, A., Domingo, D., Fitch, M., Hatton, S. & Trowbridge, I. S. (1993) *J. Biol. Chem.* **268**, 21686–21692.
19. Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A. & von, F. K. (1990) *EMBO J.* **9**, 3497–3506.
20. Johnson, K. F. & Kornfeld, S. (1992) *J. Cell Biol.* **119**, 249–257.
21. Williams, M. A. & Fukuda, M. (1990) *J. Cell Biol.* **111**, 955–966.
22. Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y. & August, J. T. (1993) *J. Biol. Chem.* **268**, 1941–1946.
23. Bernard, H. U., Oltersdorf, T. & Seedorf, K. (1987) *EMBO J.* **6**, 133–138.
24. Sato, H., Watanabe, S., Furuno, A. & Yoshiike, K. (1989) *Virology* **170**, 311–315.
25. Greenfield, I., Nickerson, J., Penman, S. & Stanley, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11217–11221.
26. Meneguzzi, G., Cerni, C., Kieny, M. P. & Lathe, R. (1991) *Virology* **181**, 62–69.
27. Wu, T. C. (1994) *Curr. Opin. Immunol.* **6**, 746–754.
28. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. & Vogelstein, B. (1990) *Science* **249**, 912–915.
29. Kruisbeek, A. & Shevach, E. (1994) *Curr. Prot. Immunol.* **1**, 3.12.01–3.12.14.
30. Earl, P. & Moss, B. (1993) *Curr. Prot. Mol. Biol.* **2**, 16.16.1–16.16.7.
31. Earl, P. & Moss, B. (1993) *Curr. Prot. Mol. Biol.* **2**, 16.17.1–16.17.16.
32. Moss, B. & Earl, P. (1993) *Curr. Prot. Mol. Biol.* **2**, 16.15.1–16.15.5.
33. Wunderlich, J. & Shearer, G. (1994) *Curr. Prot. Immunol.* **1**, 3.11.01–3.11.15.
34. Mak, T. W., Rahemtulla, A., Schilham, M., Koh, D. R. & Fung-Leung, W. P. (1992) *J. Autoimmun.* **5**, Suppl. A, 55–59.
35. Carter, J. J., Yaegashi, N., Jenison, S. A. & Galloway, D. A. (1991) *Virology* **182**, 513–521.
36. Tindle, R. W., Fernando, G. J., Sterling, J. C. & Frazer, I. H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5887–5891.
37. Bennink, J. R. & Yewdell, J. W. (1990) *Curr. Top. Microbiol. Immunol.* **163**, 153–184.
38. Sarmiento, M., Glasebrook, A. L. & Fitch, F. W. (1980) *J. Immunol.* **125**, 2665.
39. Roederer, M., Bowser, R. & Murphy, R. F. (1987) *J. Cell. Physiol.* **131**, 200–209.
40. Blum, J. S., Fiani, M. L. & Stahl, P. D. (1991) *J. Biol. Chem.* **266**, 22091–22095.
41. Renfrew, C. A. & Hubbard, A. L. (1991) *J. Biol. Chem.* **266**, 21265–21273.
42. Hammond, S. A., Bollinger, R. C., Stanhope, P. E., Quinn, T. C., Schwartz, D., Clements, M. L. & Siliciano, R. F. (1992) *J. Exp. Med.* **176**, 1531–1542.

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A chimera of the cytoplasmic tail of the mannose 6-phosphate/IGF-II receptor and lysozyme localizes to the TGN rather than prelysosomes where the bulk of the endogenous receptor is found

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SUMMARY

We fused the cytoplasmic and transmembrane domains of the bovine mannose 6-phosphate/IGF-II receptor (MPR) to lysozyme, a monomeric secretory protein thought to be devoid of sorting information. When the resulting chimera (lys/MPR) was transiently expressed in COS cells or stably expressed in CV1 cells, it had a predominantly intracellular distribution in the *trans*-Golgi region, with less than 10% present on the surface. In contrast, a similar chimera containing the transmembrane and cytoplasmic domains of the low density lipoprotein receptor (lys/LDLR) was localized to the plasma membrane, even though it endocytoses efficiently. Exchanging domains between the lys/MPR and lys/LDLR chimeras indicated that the MPR cytoplasmic domain contains the information necessary to specify the intracellular localization of the chimeric molecule. This signal must be located in the membrane-proximal third of the tail, as deletion of the last 120 residues of the 163 residue tail has no obvious effect on the distribution of lys/MPR.

However, the recycling of the lys/MPR does not completely mimic that of the intact endogenous MPR, as immunofluorescence labelling shows that they are predominantly in different locations, indicating a role for the lumenal domain of the MPR in determining the steady-state distribution of the MPR itself.

Key words: *trans*-Golgi network, coated pit, sorting

INTRODUCTION

The cation-independent mannose 6-phosphate/IGF-II receptor (MPR) binds mannose 6-phosphate residues on lysosomal enzymes as these newly synthesized molecules arrive in the *trans*-Golgi network (TGN). Here the receptor is recruited into clathrin-coated pits containing the HA-1 adaptor, thus sequestering the lysosomal enzymes from bulk secretory components for efficient delivery to lysosomes. The MPR releases its ligand in an acidified endosome (prelysosome) and returns to the TGN for multiple rounds of delivery. Also, when MPR reaches the plasma membrane it is efficiently endocytosed in clathrin-coated pits containing the HA-2 adaptor (for review of coated pit components see Pearse and Robinson, 1990; Ahle et al., 1988) and recycles back through endosomes and the TGN (for reviews see Kornfeld, 1992; Kornfeld and Mellman, 1989; von Figura, 1991; Klumperman et al., 1993).

Thus, depending on the cell type, the MPR follows an unusual transport pathway that requires multiple transport signals. The MPR cytoplasmic tail has a well defined endocytosis signal $Y^{24}KY^{26}SKV^{29}$ (Lobel et al., 1989; Jadot et al., 1992). This region also apparently acts as a signal for the efficient sorting of lysosomal enzymes from the TGN separately or in combination with the C-terminal residues $LLHV^{163}$ (Johnson and Kornfeld, 1992). Both types of coated vesicle adaptor have been shown to bind to the MPR tail. Recognition by the HA-2 plasma membrane adaptor is abolished by mutation of the tyrosines important for the endocytosis signal but these same mutations do not appear to abolish sorting (Johnson and Kornfeld, 1992) or the binding of the HA-1 Golgi adaptor (Glickman et al., 1989).

In order to see if the cytoplasmic tail could specify the intracellular distribution of a heterologous protein, we decided to construct chimeric molecules by fusing portions of the MPR to lysozyme, a protein not expected to have dominant sorting signals. Chicken lysozyme was chosen because it is monomeric, readily secreted when expressed in heterologous systems, able to tolerate C-terminal extensions and provides a convenient antigenic tag (Kondor-Koch et al., 1985; Munro and Pelham, 1987).

We expressed the hybrid proteins, and truncated or mutated derivatives, in cultured cells and looked at their steady-state distribution by immunofluorescence. In contrast to a similar hybrid protein containing lysozyme and the cytoplasmic portion of the low density lipoprotein receptor (lys/LDLR) found largely on the plasma membrane, the lysozyme/MPR hybrid (lys/MPR) was located predominantly in an intracellular compartment, most likely the TGN. However, the localization of lys/MPR did not closely mimic that of the intact, endogenous MPR found largely in prelysosomes.

MATERIALS AND METHODS

Construction of vectors for the expression of chimeras

COS cell expression vectors SAY1 (Munro and Pelham, 1987) and HYK (Pelham et al., 1988) containing the chicken lysozyme coding sequence and related vectors were kindly provided by S. Munro and H. Pelham in this laboratory. The expression vector CDM8 (Seed, 1987) was used to create stable cell lines in combination with RSV neo carrying the neomycin resistance gene (de Wet et al., 1987).

lys/LDLR and lys/MPR

The pL3 plasmid, containing cDNA encoding the human LDLR, was obtained from the American Type Culture Collection (ATCC no. 57004/57005). The complete LDLR coding sequence was cleaved from pL3 using a 5′ *Hin*dIII site and a *Sma*I site located in the 3′ untranslated sequence (bp 2818), and ligated into pHYK that had been cut with *Hin*d III and *Sma*I. The resulting vector, pLDLR, directs the expression of the human LDLR in COS cells.

A plasmid containing sequence 3500-4647 of the bovine MPR inserted into the *Sac*I site of pUC19 (pE5; Lobel et al., 1989) was kindly provided by S. Kornfeld, Washington University, St Louis, MS).

Fusion of lysozyme and receptor sequences took advantage of the termination codon of the lysozyme cDNA in pSAYI forming part of the *Xba*I site. By cutting with *Xba*I and blunting with mung bean nuclease, the stop codon as well as one other base at the 3′ end of the coding sequence is removed. *Bam*HI sites at position 2337 in the LDLR sequence and at position 3811 in the MPR sequence were created by site-directed mutagenesis in M13mp18 by the method of Zoller and Smith (1984). When these *Bam*HI sites were cut and blunted with mung bean nuclease, an extra base was left at their 5′ ends. This allowed fusion of the lysozyme sequence with each receptor sequence, restoring the last residue of the lysozyme sequence and maintaining the reading frame. Thus fragments carrying receptor sequences cut with *Bam*HI, treated with mung bean nuclease and cut with *Eco*RI were ligated into SAYI that had been cut with *Xba*I, blunted, and cut with *Eco*RI. The resulting vectors, pl/M and pl/L, are able to direct the expression of lys/MPR and lys/LDLR chimeras in COS cells. Further mutations or stop codons were introduced by site directed mutagenesis by the method of Kunkel (1985) or by PCR (Clackson, 1991). Mutated fragments were sequenced, either in M13mp18 or pUC before inserting into SAYI.

pCDM8 expression vectors

Fragments encoding lys/MPR and mutants were released from pl/M with *Hin*dIII and *Not*I and ligated into pCDM8 that had been similarly cut. The lys/LDLR coding sequence was released from pl/L as a *Hin*dIII-*Sma*I fragment. It was ligated into pCDM8, which had been cut with *Pst*I, end-filled, and cut with *Hin*dIII (thus destroying the *Pst*I site). The expression vectors thus created are pCDM8 (l/M), pCDM8 (Δ2Y), pCDM8 (1401), pCDM8 (1355), pCDM8 (1333) and pCDM8 (l/L).

Transfection of tissue culture cells

Cells were grown at 37°C under 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Transient transfection was achieved by a procedure involving calcium phosphate precipitation of the DNA. COS7 cells to be transfected were plated the night before to 50-70% confluence, and given fresh medium 1 hour before transfection. For each 75 cm^2 flask, 25 μg of PEG-purified DNA and 63 μl of 2.5 M $CaCl_2$ was added to 0.563 ml of TE/10 (1 mM Tris-HCl, pH 8.0, 0.01 mM EDTA). While bubbling air through the solution with a pasteur pipette, 0.625 ml of 2× HBS (25 mM HEPES, pH 7.12, 1.5 mM Na_2HPO_4, 280 mM NaCl) was added dropwise, then left for 20 minutes at room temperature to complete the precipitation. This was added to the tissue culture medium, and cells were gassed and incubated overnight. The cells were then rinsed with DBPS, given fresh medium, and processed the following day e.g. for immunofluorescence studies.

Alternatively, cells were transiently transfected in the presence of chloroquine. Typically, cells were grown to 75% confluence in a 25 cm^2 flask. After washing the cells twice with serum-free DMEM, 0.5 ml of serum-free DMEM and 5 μl of plasmid DNA (approx 0.5 mg/ml) were added to the flask, followed by 0.5 ml of 1 mg/ml DEAE-dextran in TBS. After a 30 minute incubation, the medium was replaced with DMEM containing 10% FCS and 100 μg/ml chloroquine, and the cells were incubated for a further 3 hours. The cells were incubated for 2 days in DMEM + 10% FCS before harvesting. Such cells were used for endocytosis assays.

For the selection of stable transfectants,the calcium phosphate technique was used to co-precipitate 20 μg of pCDM8 expression vector with 1 μg of pRSVneo. The precipitate was added to a 75 cm^2 flask of CV-1 cells (one flask per plasmid) and left overnight. The cells were washed and given fresh medium the next day (day 1); on day 2 they were split 1:5 into 100 cm^2 plates, and on day 3 the cells were put under Geneticin G418 selection (Gibco; 400 μg/ml active concentration). The G418-containing medium was replaced every 3 days, and resistant colonies picked 20 days following transfection. Some transfected cell lines were subcloned by limiting dilution (Harlow and Lane, 1988). The chimeric proteins were found to have long half lives in CV1 cells. Metabolic labelling of cells was performed with a pulse of [^{35}S]methionine followed by continued incubation in the presence of unlabelled methionine. Calculating the rates of degradation from densitometry of immunoprecipitated bands at different time points gave estimated half lives of 10-20 hours for each of the chimeras lys/1333, lys/1355 and lys/MPR.

Antibodies

The following monoclonal antibodies were used: D1.3, anti-chicken egg white lysozyme (Amit et al., 1986) a mouse IgG1,κ, provided by Jeff Foote; F10 (F10-6-18), anti-chicken egg white lysozyme, provided by R. Poljiak and Sean Munro; 1B5, a mouse IgG, which recognizes a lumenal epitope on an unidentified transmembrane protein that appears to be found in prelysosomes and lysosomes (provided by Mark Marsh, MRC Laboratory for Molecular Cell Biology, University College London); C7, anti-LDLR (Beiseigel et al., 1982), which recognizes a lumenal epitope near the LDL binding site and was provided by E. Gheradi; and 9E10, mouse monoclonal, which recognizes a peptide epitope from human c-myc (Munro and Pelham, 1987) provided by M. Lewis.

Also the following polyclonal antisera were used: rabbit anti-lysozyme, raised against chicken egg white lysozyme was provided by M. Lewis; anti-JG2 (rat 4.1 and rabbit 130), raised against a fusion protein containing the bovine MPR cytoplasmic domain (Glickman et al., 1989). It recognizes the bovine MPR, but shows little if any cross-reactivity to the human or simian MPR and staining is only seen in cells expressing the bovine MPR tail sequence; rabbit anti-galactosyltransferase, raised against human β-1,4-galactosyltransferase, provided by Eric Berger (Roth and Berger, 1982); and rabbit anti-MPR (286), raised against the bovine MPR, purified from liver (Glickman et al., 1989). It recognizes human, simian, bovine and rodent MPR. Only the affinity-purified antiserum recognizes the MPR in simian cells fixed with methanol, although the staining pattern is the same with crude antiserum and formaldehyde fixations.

Sheep anti-mouse (as well as donkey anti-rabbit) antibodies conjugated to either fluorescein isothiocyanate (FITC) or Texas Red (TR) were obtained from Amersham. These antibodies are affinity-purified and absorbed against human, rat and rabbit (or mouse) IgG, so they show no cross-reactivity and can be used for double labelling studies.

FITC-conjugated goat anti-rat IgG (Sigma) was run through a rabbit IgG column to remove any cross-reacting antibody. It recognizes both mouse and rat IgG.

Immunofluor scence microsc py

Cells were split onto sterile 4-well coated microscope slides (Hendley-Essex) and allowed to spread overnight in a drop of medium before being rinsed quickly in DPBS and fixed in one of three ways:

(1) glutaraldehyde fixation. The slides were immersed in a solution of 2% paraformaldehyde, 0.1% glutaraldehyde in PBS at RT for 30 minutes, followed by permeabilization for 10 minutes with 0.5% Triton X-100. Free aldehydes were blocked by a 10 minute incubation in 1 mg/ml $NaBH_4$ in PBS. The slides were rinsed with PBS between each step, and stored at 4°C overnight in PBS if necessary.

(2) Paraformaldehyde fixation. Slides were immersed in a freshly prepared solution of 4% paraformaldehyde in PBS for 30 minutes at RT, rinsed in PBS, then permeabilized for 10 minutes with 0.5% Triton X-100.

(3) Methanol/acetone fixation. Cells were fixed in methanol for 5 minutes at −20°C, followed by acetone for 30 seconds at −20°C, and either rinsed in PBS for immediate use or air dried and stored at −20°C.

Double label immunofluorescence was performed essentially as described by Harlow and Lane (1988). Each well of a multiwell slide was preincubated for 20 minutes in blocking buffer (either 20% FCS, 0.5% Tween-20 in PBS or 2% dried skim milk, 0.1% Tween-20 in PBS), treated with primary antibody diluted in blocking buffer for 45 minutes at RT, and washed for 15 minutes. A second primary antibody, raised in a different species from the first, was applied and incubated 45 minutes at RT. After washing, a mixture of fluorochrome-labelled secondary antibodies was applied for 45 minutes at RT, and the slides were washed extensively before mounting in 90% glycerol, 50 mM Tris, pH 8.0, containing 1 mg/ml *p*-phenylene diamine to reduce bleaching. Slides were examined on an MRC-600 laser scanning confocal microscope and the images recorded on an optical disc.

Surface/intracellular binding

The method used was modified from that of Johnson et al. (1990). Cells were grown to near confluence in 6-well plates. To estimate the amount of cell surface binding sites, the cells were chilled to 4°C, washed with DPBS+1% BSA, then incubated with ^{125}I-labelled antibody in DPBS+1% BSA for two hours at 4°C. After washing, the cells were harvested in 0.1 M NaOH, transferred (together with cell debris) to a Luckham tube and the counts measured in a gamma counter.

To estimate the total number of binding sites, cells in replicate plates were permeabilized before being incubated with ^{125}I-labelled antibody. Because washing with detergent leads to the loss of cells from the plate, the cells were first fixed with glutaraldehyde, permeabilized and treated with $NaBH_4$ (as described above), before being processed in parallel with the non-permeabilized cells.

Endocytosis assays

Endocytosis of the hybrid proteins was detected in transiently transfected COS7 cells essentially by the method of Bretscher and Lutter, 1988. Portions of packed cells (100 μl) were first labelled with 5 mg/ml of the reagent biotin-3, 3′-dithiobis(sulphosuccinimidyl propionate). To measure endocytosis, labelled cells were put into a small volume of DPBS/FCS and split into three equal portions: (1) one was held at 0°C; (2) one was held at 0°C and, in parallel with the third sample, treated with cysteine; (3) the third sample was diluted into 7 volumes of DPBS/FCS at 37°C, held there for 20 minutes with occasional shaking, chilled to 0°C, spun out and resuspended in a small volume of DPBS/FCS at 0°C. Cysteine stripping was performed as described by Bretscher (1992) in a modification of the original reduction method and cell lysates made as described, in 2% NP40. Lysozyme chimeras were immunoprecipitated on 10 μl of Sepharose to which antibody F10 had been coupled. The immunoSepharose was eluted in non-reducing SDS sample buffer at 50°C and analyzed by electrophoresis on a gradient 5-20% polyacrylamide gel, which was then blotted onto nitrocellulose. The biotin-containing proteins were detected by labelling the blot with ^{125}I-streptavidin (Amersham, UK) and the blot was autoradiographed at −70°C on flashed film for 1 to 3 days. Comparison of the label present in gel bands was done directly by counting excised bands in a gamma counter.

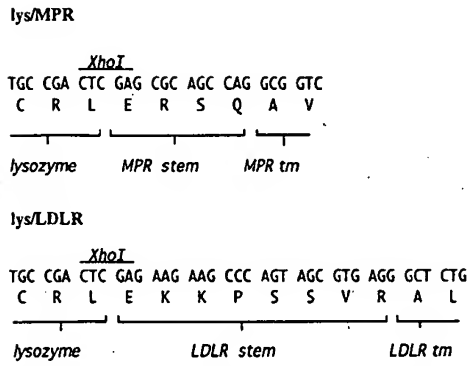

Fig. 1. Sequences at the junctions between lysozyme and receptor domains in the lys/MPR and lys/LDLR chimeras. The last residue of chicken lysozyme is fused either to residue 1271 of the bovine MPR or to residue 760 of the human LDLR. Regions of the MPR and LDLR 'stem' (i.e. residues N-terminal to the transmembrane domain) were included to provide a spacer between lysozyme and the membrane.

RESULTS

Chimeric proteins

A series of chimeras was constructed where the transmembrane and cytoplasmic domains of the MPR were fused to lysozyme, a secretory protein not normally found in COS cells.

A chimeric 'lys/MPR' molecule was created by fusing the transmembrane and cytoplasmic domains of the bovine MPR to the C terminus of chicken lysozyme, leaving 4 residues of the MPR lumenal domain to provide a spacer between lysozyme and the membrane. Similarly, a hybrid lysozyme-LDLR molecule (lys/LDLR) was created by replacing most of the human LDLR extracellular domain with lysozyme, leaving an 8 residue spacer followed by the complete transmembrane and cytoplasmic domains of the LDLR (Fig. 1). Additional chimeras were made by exchanging sequences coding for the cytoplasmic domains between lys/MPR and lys/LDLR using a PCR-based strategy, and various truncated or mutant forms of the MPR tail were created by introducing stop codons or point mutations using site-directed mutagenesis. No changes were made to the amino acid sequences (other than those described here) as a result of the cloning procedures.

The various chimeras are shown in Fig. 2. The first four (lys/LDLR, lys/MPR, lys/L/M and lys/M/L) are designed to compare the abilities of the cytoplasmic and transmembrane domains of the MPR and the LDLR to determine the intracellular distribution of lysozyme. The two tyrosines critical for endocytosis ($Y^{24}Y^{26}$) are replaced with alanine and valine ($A^{24}V^{26}$) to create the internalization-deficient lys/Δ2Y mutant. Truncation of lys/MPR after residues 1401, 1355, 1333, 1305 or 1301 creates proteins that contain 105, 59, 35, 9 or 5 aa of the 163 aa MPR tail, respectively. However a

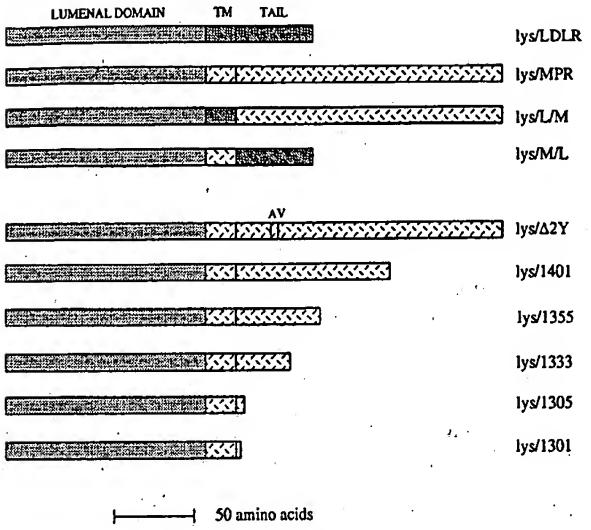

Fig. 2. Schematic representation of the chimeric constructs. The chimeras are shown as rectangles, with the N-termini on the left, and divided into sections corresponding to the lumenal, transmembrane (TM) or cytoplasmic (TAIL) domains. Sections are shaded to indicate domains derived from lysozyme (light dots on left), MPR (hatched) or LDLR (darker dots). For chimeras with cytoplasmic truncations, the last residue of the bovine MPR sequence present in the construct is given in the name e.g. lys/1401. The mutations shown in lys/Δ2Y convert Y24 to alanine and Y26 to valine.

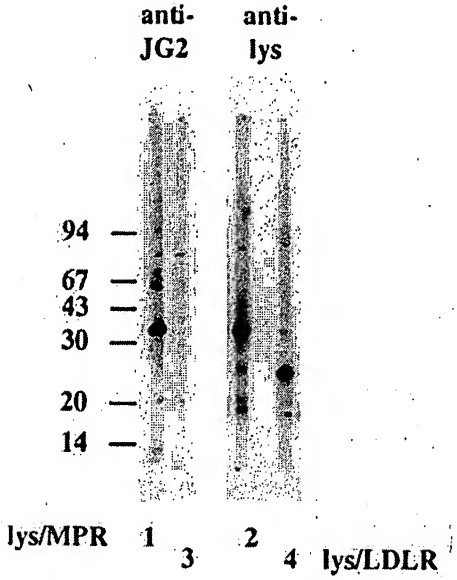

Fig. 3. Expression of chimeric proteins in COS cells. Proteins from COS cells transfected with either lys/MPR or lys/LDLR were separated on a 5-25% SDS-polyacrylamide gel and blotted onto nitrocellulose. Replicate blots were probed with either a rabbit anti-lysozyme antiserum or a rat anti-JG2 antiserum (followed by rabbit anti-rat IgG) and detected with ^{125}I-Protein A. The sizes of the labelled bands are consistent with the predicted molecular masses of the chimeric proteins. Size standards (kDa) are shown on the left.

variant of lys/1333 has since been found that codes for additional residues 36 to 39 and a further repeat of residues 32 to 35 (i.e. 8 further residues) before the expected stop codon.

A SAY1-based plasmid containing the SV40 origin of replication was used to direct the expression of chimeric proteins in COS cells under the control of the adenovirus major late promoter (Munro and Pelham, 1987). This plasmid replicates to high copy number in COS7 cells, a monkey fibroblast line, derived from CV1 cells, which contains an integrated copy of the SV40 T antigen gene, allowing a high level of expression of target sequences (Mellon et al., 1981).

Expression in COS cells

The lys/MPR and lys/LDLR chimeras, when expressed in COS cells and analysed by SDS-PAGE followed by immunoblotting with an anti-lysozyme antiserum, are of the expected size (Fig. 3). Furthermore, an antiserum raised against a fusion protein that contains the bovine MPR cytoplasmic domain (anti-JG2; Glickman et al., 1989) recognizes lys/MPR, but does not recognize the endogenous COS cell MPR or lys/LDLR (Fig. 3). Truncated and mutant forms of lys/MPR also behave as proteins of the expected size and are recognized by both anti-lysozyme and anti-JG2 antibodies (not shown), with the exception of chimeras containing 35 residues or less of the MPR tail. These mutants are not recognized by the anti-JG2 antiserum, possibly because the membrane-proximal portion of the MPR tail is highly conserved between mouse, cow and man.

Differential distribution of lys/MPR and lys/LDLR

The localization of the lys/MPR and lys/LDLR chimeras when expressed in COS cells was examined by indirect immunofluorescence microscopy of methanol/acetone fixed cells. As shown in Fig. 4, lys/MPR is found primarily in an intracellular compartment with a perinuclear, reticulate morphology characteristic of the TGN, as well as in many small vesicles distributed throughout the cytoplasm that are more numerous in highly-expressing cells. In contrast, lys/LDLR shows a diffuse plasma membrane staining similar to that seen when the intact LDLR is expressed in COS cells and stained with anti-LDLR mAb C7. A four hour cycloheximide treatment did not change this distribution (not shown), and lys/MPR staining was the same using antibodies to either the lumenal or cytoplasmic domains, indicating that the hybrid protein is intact. Neither chimera is retained in the ER, a common fate of misfolded proteins. Disruption of the MPR tail internalization signal results in a chimeric protein (lys/Δ2Y) that is found predominantly at the plasma membrane. This implies that lys/MPR is recycling in the cell and not simply retained in the Golgi, as failure to endocytose has been shown to result in the surface accumulation of MPR that is recycled through the plasma membrane (Lobel et al., 1989).

To confirm that these constructs are actually present on the plasma membrane, the chimeras were tested for their accessibility to mAb F10, which was added at 4°C to the medium of transfected, nonpermeabilised cells. The proportion of chimeric receptor found on the surface was estimated by binding iodinated antibodies in the presence and absence of detergent (Fig. 5). After subtraction of background binding observed on non-transfected cells, only 6% of lys/MPR, but

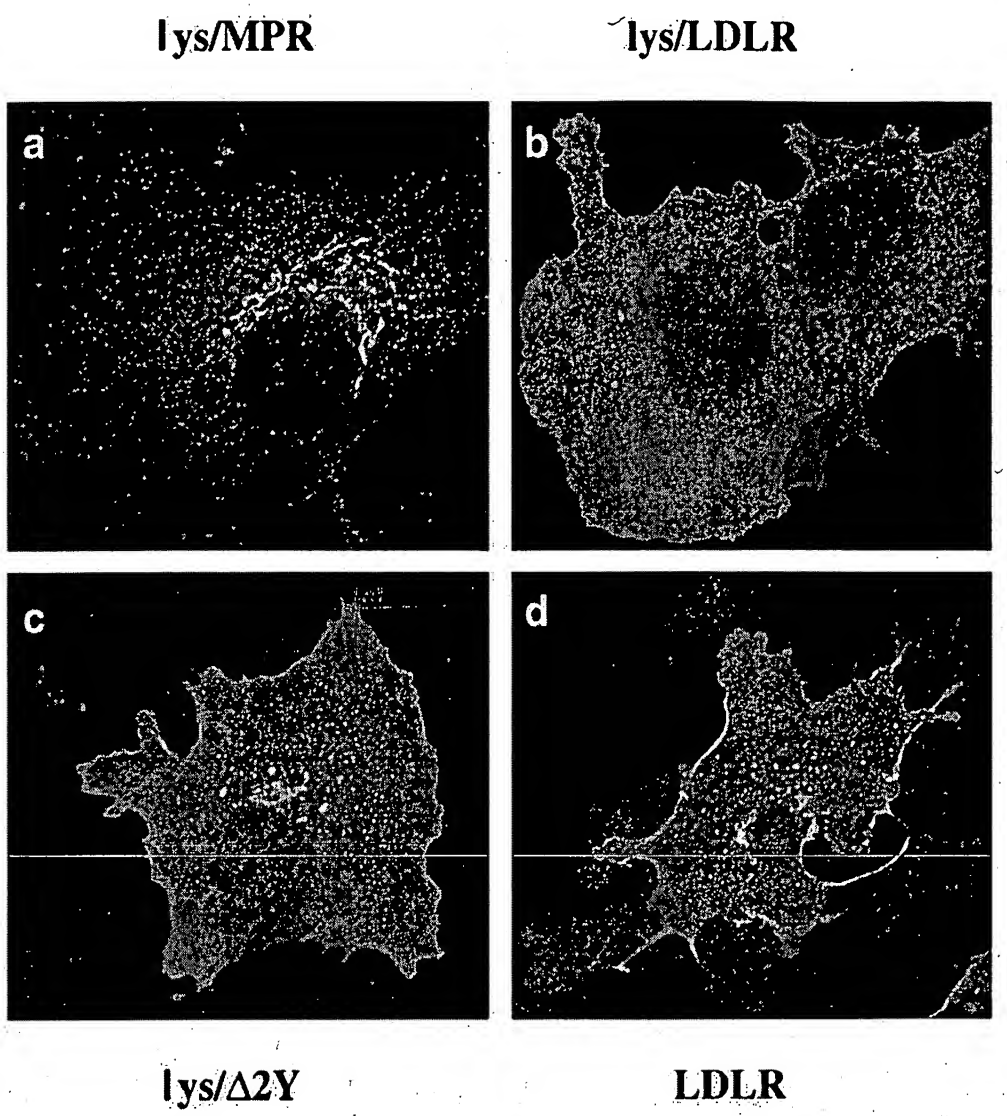

Fig. 4. Immunofluorescent localization of lys/MPR, lys/LDLR, lys/Δ2Y and human LDLR expressed in COS cells. Indirect immunofluorescence of methanol/acetone-fixed cells using either anti-lysozyme mAb D1.3 (a,b,c) or anti-LDLR mAb C7 (d) followed by fluorescein-conjugated second antibody. The lys/MPR chimera is found primarily in intracellular structures rather than on the plasma membrane, whereas labelling of the plasma membrane can be readily seen for lys/LDLR, the internalization-deficient lys/Δ2Y and for the human LDLR.

83% of the lys/LDLR and 75% of the lys/Δ2Y, was located at the plasma membrane.

Fig. 6 shows that the cytoplasmic domains rather than the transmembrane regions determine the distribution of the hybrid molecules. Lys/L/M with the LDLR transmembrane segment has an intracellular localization comparable to that of lys/MPR, while lys/M/L, like lys/LDLR, is found on the surface. Deletion of most of the MPR tail, leaving either 3 or 7 residues to provide a membrane anchor (lys/MPR 1301 and lys/1305, respectively), results in considerable ER retention. Proteins that are not well folded or correctly positioned in the membrane are often retained in the ER (Hurtley and Helenius, 1989), and both lys/1301 and lys/1305 have C-terminal sequences reminiscent of the 'KKXX' ER retention signal (Nilsson et al., 1989) (ie. lys/1301: KKX; lys/1307: RRXX). Nevertheless, immunofluorescent labelling of transfected cells at 4°C shows that both lys/1301 and lys/1305 are also expressed on the surface (not shown, but see next section for surface labelling of lys/1301).

Thus the MPR cytoplasmic tail specifies the intracellular localization of lys/MPR. All chimeras with truncated MPR tails that retain an intact internalization signal (lys/1333, lys/1355 and lys/1401) are found intracellularly, and their distribution is indistinguishable from those with a full length MPR tail (for an example see Fig. 7 for lys/1333 expression in stably transfected CV1 cells).

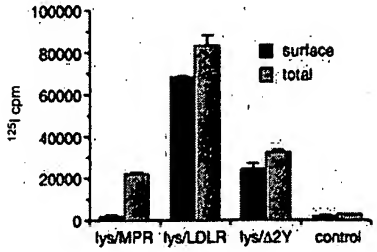

Fig. 5. Cell surface expression of lys/LDLR, lys/MPR and lys/Δ2Y in COS cells. ^{125}I-F10 binding to non-permeabilized (surface) or fixed and permeabilized cells (total) expressing each of the three chimeras, with binding to non-transfected cells serving as a control. The average of triplicate determinations is shown.

Ability of chimeras to be endocytosed

To show that the lysozyme chimeras recycle via the plasma membrane, the capacity of lys/LDLR molecules to undergo endocytosis was observed. Cell surface proteins were labelled with biotin and endocytosed molecules were detected essentially by the method of Bretscher and Lutter (1988). The results are shown in Fig. 8.

Those hybrid molecules that are largely present on the cell surface (lys/LDLR and lys/1301) are readily labelled with biotin-3,3′-dithiobis(sulphosuccinimidyl propionate) as shown in Fig. 8 (lanes d and g). The labelled proteins are extracted from cell extracts by immunoprecipitation with the F10 monoclonal antibody bound to Sepharose, fractionated on an SDS gel, blotted onto nitrocellulose and detected with ^{125}I-streptavidin. A major band of the appropriate molecular size is obvious for both constructs lys/LDLR and lys/1301 (lanes d and g) whereas none is visible in a parallel transfection with no construct (lane a). These labelled bands disappear when the cells are reduced prior to extraction (lanes e and h). However, when the cells are warmed for 20 minutes at 37°C, allowing endocytosis to take place, labelled, internalized membrane proteins are protected from the reduction process. Lys/LDLR is evidently endocytosed efficiently (lane f) and the internal pool, almost certainly saturated after 20 minutes at 37°C (Bretscher and Lutter, 1988), of the lys/LDLR construct is up to about 20%. By this criterion most of the lys/LDLR molecules are on the cell surface at steady state, in agreement with the estimate from the antibody labelling experiment. Lys/1301 is only marginally protected by warming the cells to 37°C for 20 minutes. Most of the hybrid (90% or more) is essentially trapped on the cell surface as the protein lacks a signal for efficient coated pit endocytosis, though a limited degree of internalization occurs.

Unfortunately, there was too little of hybrids lys/MPR and lys/1333 on the cell surface to label adequately in order to measure endocytosis satisfactorily. To provide evidence of the recycling of lys/MPR, its ability to endocytose F10 antibody at 37°C was tested. Cells transfected with lys/MPR are able to take up and internalize the F10 antibody, bound to lys/MPR (Fig. 9). In contrast, no internalized antibody could be seen in control cells incubated at 4°C (not shown).

Differential distribution of lys/MPR and the endogenous MPR in stable cell lines

The chimeras lys/MPR, lys/1401, lys/1355 and lys/1333 in stably expressing CV1 cell lines all show the same characteristic perinuclear, reticulate staining pattern (see Fig. 7). Little surface staining was evident with these constructs compared to the surface staining in stable lines expressing reasonably high levels of lys/LDLR and lys/Δ2Y (not shown).

Labelling of lys/MPR (and also lys/1333 and lys/1355) shows considerable, but not precise, overlap with galactosyl transferase, an enzyme restricted to the medial Golgi (Fig. 10). This indicates that the constructs recycle to somewhere in the Golgi region, most likely the TGN (Duncan and Kornfeld, 1988; Snider and Rogers, 1985), however the various cisternae and the TGN cannot be resolved by fluorescence microscopy.

In rat hepatoma cells, MPR has been shown by immunoelectron microscopy to be present in coated pits in the TGN but also to be clustered into interior membranes in the prelysosome compartment (Klumperman et al., 1993).

Typically, the MPR is found in the TGN or prelysosomes, although its relative distribution between the various locations along its recycling route varies between cell types (Griffiths et al., 1988). In a bovine fibroblast primary cell line, the MPR is almost entirely confined to the TGN, where it colocalizes with gamma adaptin (Glickman et al., 1989). In contrast (Fig. 11), the endogenous MPR in CV1 cells is seen almost exclusively

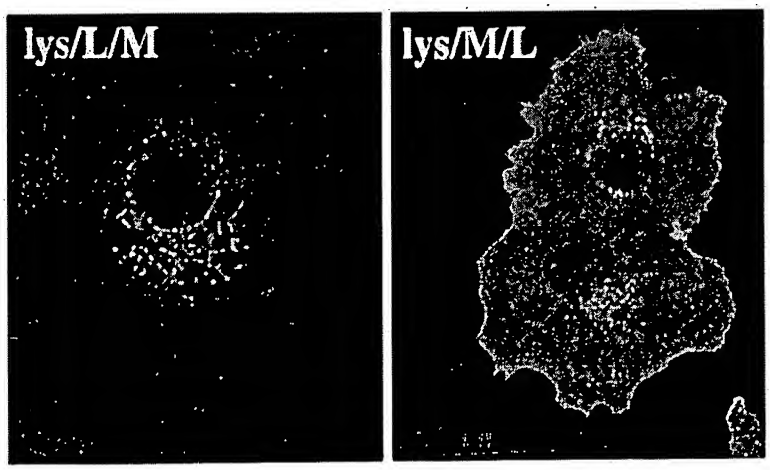

Fig. 6. The cytoplasmic domain of the MPR is required for its intracellular localization. Indirect immunofluorescence of glutaraldehyde-fixed COS cells expressing either lys/L/M (LDLRtm +MPR tail) or lys/M/L (MPR tm + LDLR tail) using mAb F10. The distribution of lys/L/M is similar to that of lys/MPR, whereas lys/M/L has the same distribution as lys/LDLR.

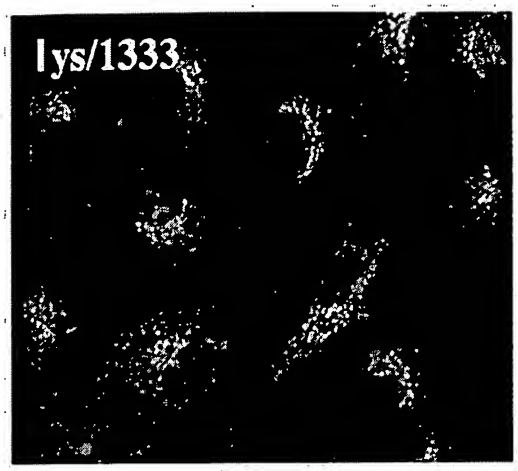

Fig. 7. Indirect immunofluorescence of CV1 cells stably transfected with the lys/1333 construct. Cells were subjected to methanol/acetone fixation and labelled with anti-lysozyme mAb D1.3 and an FITC-conjugated second antibody. A low magnification view of a confluent monolayer is shown to illustrate the degree of homogeneity of expression. Similar patterns of expression were seen for constructs lys/1355 and lys/1401 and lys/MPR.

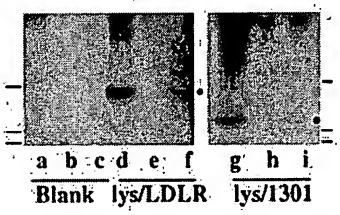

Fig. 8. Endocytosis of hybrid molecules. Autoradiographs showing the indicated lysozyme chimeras from transfected COS cells. The lysozyme hybrids were isolated on an F10 antibody-Sepharose column, from cells labelled at 0°C with biotin, run on an SDS gel, blotted and detected with ^{125}I-streptavidin. The first lane of each set of three samples (a,d and g) represents total isolated labelled material. The second lane of each set (b,e and h) is material from cells labelled and reduced at 0°C. The third lane of each set (c,f and i) shows lysozyme chimera (except c, which was a blank transfection with no construct) from cells labelled at 0°C, incubated at 37°C to allow endocytosis of biotinylated surface material, then reduced at 0°C. Markers are indicated representing molecular masses of 12 kDa, 17 kDa and 30 kDa, respectively.

in vesicular structures, likely to be prelysosomes, which double label with the monoclonal antibody 1B5. This distribution is clearly distinct from that of the lys/MPR chimera and is not affected by the latter's coexpression. Therefore, although the MPR tail is sufficient to specify the intracellular retention of the chimeric protein, the lys/MPR hybrid does not have exactly the same recycling behaviour as the intact MPR in CV1 cells, even though it clearly does recycle as shown by its ability to endocytose bound F10 antibodies (Fig. 9).

DISCUSSION

We have created chimeric proteins consisting of lysozyme, a transmembrane region and the cytoplasmic tail of either MPR

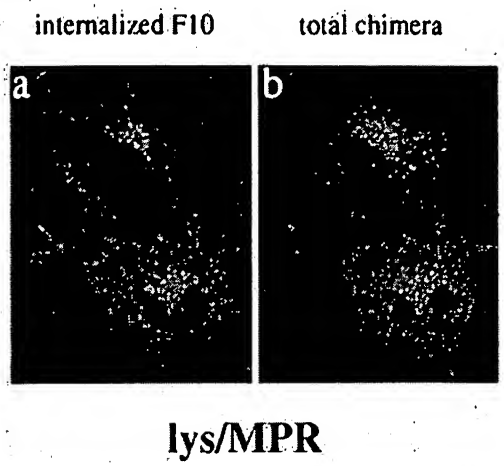

Fig. 9. Antibody uptake by lys/MPR transiently expressed in COS cells. Transfected cells were allowed to endocytose mAb F10 added to the medium (at 25 μg/ml) for 1 hour at 37°C. Internalized antibody was detected by labelling the glutaraldehyde-fixed and permeabilized cells with FITC-conjugated anti-mouse IgG antiserum (shown on the left) and the chimeric proteins were detected by double-labelling with a rabbit anti-lysozyme antiserum (shown on the right).

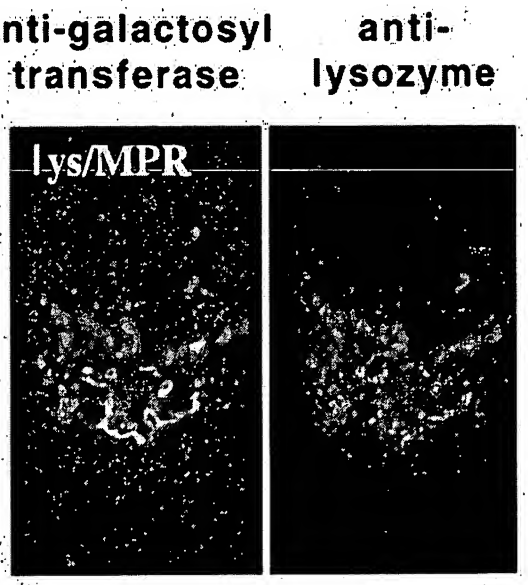

Fig. 10. Intracellular distribution of lys/MPR compared with a Golgi marker by double label immunofluorescence. The two panels show the perinuclear region of a single CV1 cell fixed in methanol/acetone and double labelled with rabbit anti-galactosyl transferase antiserum (on the left) and mAb D1.3 (on the right). The Texas Red- and fluorescein-conjugated secondary antibodies were detected simultaneously using a laser scanning confocal microscope.

(lys/MPR) or LDLR (lys/LDLR). We believe that these hybrid molecules circulate through various membrane compartments in the cell as has been shown for MPR and other endocytosed molecules (Duncan and Kornfeld, 1988; Stoorvogel et al., 1989; Green and Kelly, 1992). We have expressed the chimeric

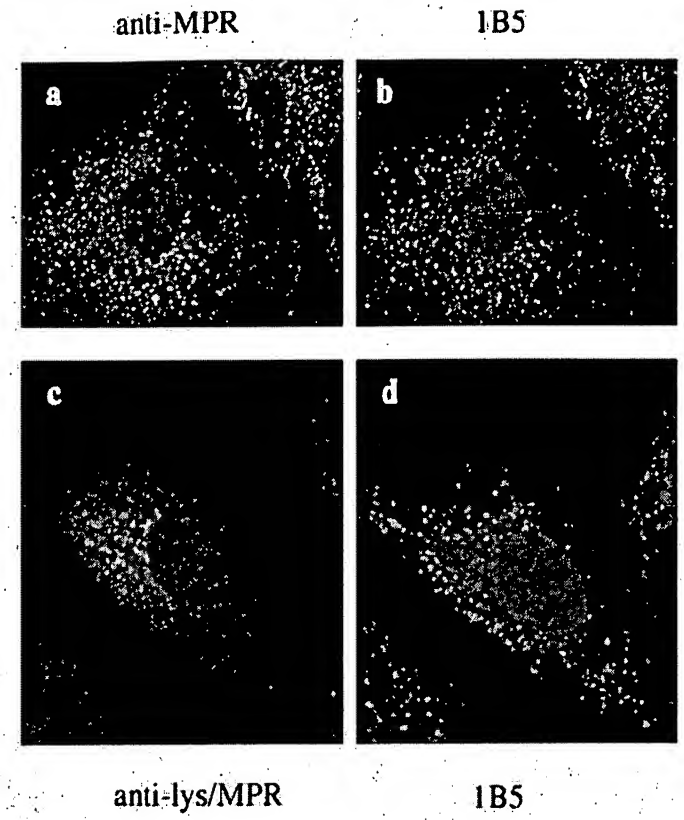

Fig. 11. Comparison of the subcellular distribution of lys/MPR with that of the endogenous MPR. (a and b) In CV1 cells, the endogenous MPR is found in scattered vesicles that can also be labelled with the prelysosomal/lysosomal marker 1B5. In a lys/MPR cell line, the endogenous MPR colocalizes with 1B5 as above. However, anti-bovine MPR tail (anti-JG2) labelling of lys/MPR is clearly distinct from the vesicular staining pattern seen with mAb 1B5 (c and d). We did not try to double label the same cells for MPR and lys/MPR as our antibody against MPR was raised against the whole receptor.

proteins in COS cells or CV1 cells and taken a snapshot view of their steady-state distribution by immunofluorescence microscopy, in order to show the location in which they spend most of their time. We found the lys/MPR hybrid largely in an intracellular compartment in the Golgi region, probably corresponding to the TGN. Dominant signals on lysozyme are not responsible for this distribution, as the lysozyme-LDLR hybrid was found on the cell surface. The membrane-spanning region is known to be important for the Golgi retention of glycosyl transferases (Munro, 1991; Nilsson et al., 1991; Teasdale et al., 1992; Bretscher and Munro, 1993), but exchanging transmembrane domains between these chimeras shows that the MPR tail, and not the transmembrane domain, is responsible for the intracellular distribution of the lys/MPR protein. By expressing truncated forms of lys/MPR, the signal required for intracellular retention was mapped to the first 39 aa of the MPR tail.

The dominant sorting feature identified so far in the membrane proximal third of the MPR tail is the sequence (Y^{24} KY^{26} SKV^{29}) apparently containing overlapping but distinct signals for endocytosis by plasma membrane coated pits and lysosomal enzyme sorting mediated by TGN coated pits (Johnson and Kornfeld, 1992). The signal for endocytosis depends chiefly on the spaced hydrophobic residues Y^{24}, Y^{26} and V^{29} (Lobel et al., 1989; Jadot et al., 1992) correlating with the observation that the two tyrosine residues are important for the interaction of the plasma membrane adaptor (HA-2) with the MPR tail (Glickman et al., 1989). However, lysosomal enzyme sorting is minimally altered in vivo when tyrosines 24 and 26 are substituted with alanines and other features and regions of the tail such as the C-terminal residues $LLHV^{163}$ play a role in this process (Johnson and Kornfeld, 1992). Substitution of the two tyrosines does not appear to affect binding of the Golgi adaptor (HA1) to the MPR tail either, indicating that other regions of the tail are involved in efficient Golgi adaptor binding, likely also to be necessary for lysosomal enzyme sorting in vivo. However, the precise nature of Golgi adaptor binding sites has still to be determined.

An interesting parallel can be drawn between the behaviour of lys/MPR and a hybrid protein containing the cytoplasmic tail of TGN38 (Luzio et al., 1990; Bos et al., 1993; Humphrey et al., 1993). In this case also, a short, tyrosine-containing sequence within the cytoplasmic tail is sufficient to confer TGN-localization to a reporter protein Tac. This sequence acts as an efficient endocytosis signal dependent on the usual spacing of hydrophobic residues. However, an R335D mutation in the sequence still allows the mutant to undergo efficient endocytosis but abolishes its ability to localize to the TGN (Humphrey et al., 1993). Presumably this mutation allows a greater proportion of the Tac hybrid to cycle via the plasma membrane thus reducing the amount seen in the TGN at steady state, a situation perhaps closer to that observed with the lys/LDLR construct.

The steady-state localization of the MPR varies between the TGN and prelysosomes in different cell types and may be influenced by the presence of ligand (Griffiths et al., 1988; Brown, 1990). Although the cytoplasmic tail of the MPR promotes the intracellular retention of the receptor, in CV1

cells, the lumenal domain is apparently required to allow a greater proportion of the receptor to appear in the prelysosomes rather than the TGN at steady state. Recently, Klumperman et al. (1993) have performed a careful study of MPR distribution in HepG2 and BHK cells by electron microscopy of immunolabelled cryosections. They found that the MPR exited the TGN via the HA-1 adaptor containing coated vesicles. On arrival in prelysosomes, the MPR was relatively enriched in internal vesicles of this morphologically complex compartment. From our observations, the MPR seems to be present in corresponding locations in CV1 cells. The lys/MPR construct is likely to enter Golgi coated pits with the MPR but it could well recycle between the TGN and the prelysosome with altered kinetics, resulting in a different steady-state distribution. The studies of Brown and co-workers (1986) suggest that the MPR is localized to the TGN in the absence of ligand, whereas conditions that prevent the release of bound ligand result in a redistribution of the MPR to the prelysosome. According to this model, the lys/MPR chimera would be expected to be found in the TGN at steady state as it lacks a ligand-binding domain. In fact, lys/MPR behaves similarly to the MPR in I cells, which do not attach the M6P recognition marker to lysosomal enzymes and are therefore devoid of ligand (Brown, 1990). However, a number of studies using reagents that perturb ligand binding have failed to find an effect on surface-to-endosome transport of MPR (Braulke et al., 1987; Pfeffer, 1987). Ligand binding does not appreciably affect the surface to TGN route in K562 human erythroleukemia cells, as treatments that increase (chloroquine, monensin, β-galactosidase) or decrease (M6P, cycloheximide, tunicamycin) receptor occupancy did not affect the resialylation of surface MPR treated with neuraminidase (Jin et al., 1989). Therefore, the overall recycling of MPR is not ligand dependent; nevertheless, it is intriguing to speculate that ligand, perhaps by some form of signal transduction, influences the rate of a transport step and therefore changes the steady-state distribution of the MPR at least in some cell types, including CV1 cells.

Recent studies have provided other examples of receptors that show a ligand-dependent increase in the rate of transport. The accumulation of the EGF-receptor in the multivesicular bodies of prelysosomes seems to depend on kinase activity in response to EGF (Felder et al., 1990). Examples also include the pIg receptor (Bomsel and Mostov, 1991) and the human KDEL receptor (Lewis and Pelham, 1992). If the extent of traffic along a certain pathway is responsive to the amount of ligand in the system, the efficiency of the transport mechanism would be optimized. Clearly, in the case of the MPR, ligand binding affects the conformation of the receptor's cytoplasmic tail, which alters its ability to bind to other molecules, e.g. heterotrimeric G proteins (Murayama et al., 1990) and perhaps clathrin coat proteins in some way, which may in turn affect one or more transport events. In this respect it is interesting that Méresse and Hoflack (1993) have observed that the MPR is phosphorylated on its cytoplasmic tail as it passes through the *trans*-Golgi region.

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REFERENCES

Ahle, S., Mann, A., Eichelsbacher, U. and Ungewickell, E. (1988). Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO J.* **7**, 919-929.

Amit, A. G., Mariuzza, R. A., Phillips, S. E. V. and Poljak, R. J. (1986). Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution. *Science* **233**, 747.

Beisiegel, U., Schneider, W. J., Brown, M. S. and Goldstein, J. L. (1982). Immunoblot analysis of low density lipoprotein receptors in fibroblasts from subjects with familial hypercholesterolemia. *J. Biol. Chem.* **257**, 13150-13156.

Bomsel, M. and Mostov, K. (1991). Involvement of a GTP-binding protein in sorting of the plg-receptor into transcytotic vesicles and role of the dlgA ligand. *J. Cell Biol.* **115**, 195a.

Bos, K., Wraight, C. and Stanley, K. K. (1993). TGN38 is maintained in the trans-Golgi network by a tyrosine-containing motif in the cytoplasmic domain. *EMBO J.* **12**, 2219-2228.

Braulke, T., Gartung, C., Hasilik, A. and von Figura, K. (1987). Is movement of mannose 6-phosphate-specific receptor triggered by binding of lysosomal enzymes? *J. Cell Biol.* **104**, 1735-1742.

Bretscher, M. S. and Lutter, R. (1988). A new method for detecting endocytosed proteins. *EMBO J.* **7**, 4087-4092.

Bretscher, M. S. (1992). Circulating integrins: $\alpha_5\beta_1$, $\alpha_6\beta_4$ and Mac-1, but not $\alpha_3\beta_1$, $\alpha_4\beta_1$ or LFA-1. *EMBO J.* **11**, 405-410.

Bretscher, M. S. and Munro, S. (1993). Cholesterol and the Golgi apparatus. *Science* **261**, 1280-1281.

Brown, W. J., Goodhouse, J. and Farquhar, M. G. (1986). Mannose-6-phosphate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. *J. Cell Biol.* **103**, 1235-1247.

Brown, W. J. (1990). Cation-independent mannose 6-phosphate receptors are concentrated in trans Golgi elements in normal human and I-cell disease fibroblasts. *Eur. J. Cell Biol.* **51**, 201-210.

Clackson, T., Gussow, D. and Jones, P. T. (1991). General applications of PCR to gene cloning and manipulation. In *PCR, A Practical Approach* (ed. M. J. McPherson, P. Quirke and I. R. Taylor), pp. 187-214. IRL Press, Oxford, UK.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. and Subramanis, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725-737.

Duncan, J. R. and Kornfeld, S. (1988). Intracellular movement of two mannose 6-phosphate receptors: return to the Golgi apparatus. *J. Cell Biol.* **106**, 617-628.

Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J. and Hopkins, C. R. (1990). Kinase activity controls the sorting of the epidermal growth factor receptor within the multi vesicular body. *Cell* **61**, 623-624.

Glickman, J. N., Conibear, E. and Pearse, B. M. F. (1989). Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO J.* **8**, 1041-1047.

Green, S. A. and Kelly, R. B. (1992). Low density lipoprotein receptor and cation-independent mannose 6-phosphate receptor are transported from the cell surface to the Golgi apparatus at equal rates in PC12 cells. *J. Cell Biol.* **117**, 47-55.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* **52**, 329-341.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual.* New York, Cold Spring Harbor Laboratory Press.

Humphrey, J. S., Peters, P. J., Yuan, L. C. and Bonifacino, J. S. (1993). Localization of TGN38 to the trans-Golgi network: Involvement of a cytoplasmic tyrosine-containing sequence. *J. Cell Biol.* **120**, 1123-1135.

Hurtley, S. M. and Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **5**, 277-307.

Jadot, M., Canfield, W. M., Gregory, W. and Kornfeld, S. (1992). Characterization of the signal for rapid internalization of the bovine mannose 6-phosphate/insulin-like growth factor II receptor. *J. Biol. Chem.* **267**, 11069-11077.

Jin, M., Sahagian, G. G. and Snider, M. D. (1989). Transport of surface mannose 6-phosphate receptor to the Golgi complex in cultured human cells. *J. Biol. Chem.* **264**, 7675-7680.

Johnson, K. F., Chan, W. and Kornfeld, S. (1990). Cation-dependent mannose 6-phosphate receptor contains two internalization signals in its cytoplasmic domain. *Proc. Nat. Acad. Sci. USA* **87**, 10010-10014.

Johnson, K. F. and Kornfeld, S. (1992). The cytoplasmic tail of the mannose

6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J. Cell Biol.* **119**, 249-257.

Klumperman, J., Hille, A., Veenendaal, T., Oorschot, V., Stoorvogel, W., von Figura, K. and Geuze, H. J. (1993). Differences in the endosomal distributions of the two mannose 6-phosphate receptors. *J. Cell Biol.* **121**, 997-1010.

Kondor-Koch, C., Bravo, R., Fuller, S., Cutler, D. and Garoff, H. (1985). Exocytotic pathways exist to both the apical and the basolateral cell surface of the polarized epithelial cell MDCK. *Cell* **43**, 297-306.

Kornfeld, S. (1992), Structure and function of the mannose 6-phosphate/insulin-like growth factor receptors. *Annu. Rev. Biochem.* **61**, 307-330.

Kornfeld, S. and Mellman, I. (1989). The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**, 483-525.

Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Nat. Acad. Sci. USA* **82**, 488-492.

Lewis, M. J. and Pelham, H. R. B. (1992). Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell* **68**, 353-364.

Lobel, P., Fujimoto, K., Ye, R. D., Griffiths, G. and Kornfeld, S. (1989). Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. *Cell* **57**, 787-796.

Luzio, J. B., Brake, B., Banting, G., Howell, K. E., Braghetta, P. and Stanley, K. K. (1990). Identification, sequencing and expression of an integral membrane protein of the trans-Golgi network (TGN38). *Biochem. J.* **270**, 97-102.

Mellon, P., Parker, V., Bluzman, Y. and Maniatis, T. (1981). Identification of DNA sequences required for transcription of the human alpha globin gene in a new SV40 host vector system. *Cell* **27**, 279-288.

Méresse, S. and Hoflack, B. (1993). Phosphorylation of the cation-independent mannose 6-phosphate receptor is closely associated with its exit from the trans-Golgi network. *J. Cell Biol.* **120**, 67-75.

Munro, S. and Pelham, H. R. B. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907.

Munro, S. (1991). Sequences within and adjacent to the transmembrane segment of α-2,6-sialyltransferase specify Golgi retention. *EMBO J.* **10**, 3577-3588.

Murayama, Y., Okamoto, T., Ogata, E., Asano, T., Iiri, T., Katada, T., Ui, M., Grubb, J. H., Sly, W. S. and Nishimoto, I. (1990). Distinctive regulation of the functional linkage between the human cation-independent mannose 6-phosphate receptor and GTP-binding proteins by insulin-like growth factor II and mannose 6-phosphate. *J. Biol. Chem.* **265**, 17456-17462.

Nilsson, T., Jackson, M. and Peterson, P. A. (1989). Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* **58**, 707-718.

Nilsson, T., Lucocq, J. M., MacKay, D. and Warren, G. (1991). The membrane-spanning domain of β-1,4-galactosyltransferase specifies trans Golgi localization. *EMBO J.* **10**, 3567-3575.

Pearse, B. M. F. and Robinson, M. S. (1990). Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* **6**, 151-172.

Pelham, H. R. B., Hardwick, K. G. and Lewis, M. J. (1988). Sorting of soluble ER proteins in yeast. *EMBO J.* **7**, 1757-1762.

Pfeffer, S. R. (1987). The endosomal concentration of a mannose 6-phosphate receptor is unchanged in the absence of ligand synthesis. *J. Cell Biol.* **105**, 229-234.

Roth, J. and Berger, E. G. (1982). Immunocytochemical localisation of galactosyltransferase in HeLa cells: co-distribution with thiamine pyrophosphatase in trans-Golgi cisternae. *J. Cell Biol.* **93**, 223-229.

Seed, B. (1987). An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. *Nature* **329**, 840-842.

Snider, M. D. and Rogers, O. C. (1985). Intracellular movement of cell surface receptors after endocytosis; resialylation of asialo-transferrin receptor in human erythroleukemia cells. *J. Cell Biol.* **100**, 826-834.

Stoorvogel, W., Geuze, H. J., Griffith, J. M., Schwartz, A. L. and Strous, G. J. (1989). Relations between the intracellular pathways of the receptors for transferrin, asialoglycoprotein, and mannose 6-phosphate in human hepatoma cells. *J. Cell Biol.* **108**, 2137-2148.

Teasdale, R. D., D'Agostaro, G. and Gleeson, P. A. (1992). The signal for Golgi retention of bovine β1,4-galactosyltransferase is in the transmembrane domain. *J. Biol. Chem.* **267**, 4084-4096.

von Figura, K. (1991). Molecular recognition and targeting of lysosomal proteins. *Curr. Opin. Cell Biol.* **3**, 642-646.

Zoller, M. J. and Smith, M. (1984). Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single stranded DNA template. *DNA* **3**, 479-488.

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Targeting of a lysosomal membrane protein: a tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes

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Communicated by K.von Figura

Lysosomal acid phosphatase (LAP) is synthesized as a transmembrane protein with a short carboxy-terminal cytoplasmic tail of 19 amino acids, and processed to a soluble protein after transport to lysosomes. Deletion of the membrane spanning domain and the cytoplasmic tail converts LAP to a secretory protein, while deletion of the cytoplasmic tail as well as substitution of tyrosine 413 within the cytoplasmic tail against phenylalanine causes accumulation at the cell surface. A chimeric polypeptide, in which the cytoplasmic tail of LAP was fused to the ectoplasmic and transmembrane domain of hemagglutinin is rapidly internalized and tyrosine 413 of the LAP tail is essential for internalization of the fusion protein. A chimeric polypeptide, in which the membrane spanning domain and cytoplasmic tail of LAP are fused to the ectoplasmic domain of the M_r 46 kd mannose 6-phosphate receptor, is rapidly transported to lysosomes, whereas wild type receptor is not transported to lysosomes. We conclude that a tyrosine containing endocytosis signal in the cytoplasmic tail of LAP is necessary and sufficient for targeting to lysosomes.

Key words: endocytosis signal/internalization/lysosomes/targeting

Introduction

Several proteins of the lysosomal membrane have been identified in recent years (Lewis *et al.*, 1985; Chen *et al.* 1985; Lippincott-Schwartz and Fambrough, 1986; Barriocanal *et al.*, 1986). The contribution of these proteins to the specific functions of the lysosomal membrane, such as generation and maintenance of an acidic milieu, stability towards lysosomal hydrolases, and transport of low molecular weight components into and out of lysosomes remains to be established. Structural features common to this class of membrane proteins are a high number of *N*-linked sialylated oligosaccharides, a single membrane spanning domain and relatively short carboxy-terminal cytoplasmic tails of 10–11 residues (Fambrough *et al.*, 1988; Chen *et al.*, 1988; Viitala *et al.*, 1988; Howe *et al.*, 1988). In contrast to soluble proteins of the lysosomal matrix the lysosomal membrane proteins are routed from the Golgi complex to lysosomes independently of mannose 6-phosphate receptors. Minor fractions of some of the lysosomal membrane glycoproteins are located at the plasma membrane, where their appearance can be influenced by the state of differentiation of the cell (Viitala *et al.*, 1988;) or the lysosomal pH (Lippincott-Schwartz and Fambrough, 1987). The structural features that are involved in targeting of lysosomal membrane proteins are unknown. For a growing number of membrane glycoproteins it has been shown that sorting signals are localized within their cytoplasmic tails. The trafficking of receptors for LDL (Davis *et al.*, 1986, 1987) polymeric Ig (Mostov *et al.*, 1986), EGF (Prywes *et al.*, 1986), transferrin (Rothenberger *et al.*, 1987; Jacopetta *et al.*, 1988) and mannose 6-phosphate (Lobel *et al.*, 1989) depends on signals within their cytoplasmic tails. One of these trafficking signals mediates rapid endocytosis and contains an essential tyrosine residue. Exchange of a tyrosine residue within the cytoplasmic tail of the LDL receptor by a non-aromatic amino acid impairs its integration into coated pits and internalization (Davis *et al.*, 1987). Replacement of a cysteine by a tyrosine within the cytoplasmic tail converts the influenza virus hemagglutinin from a protein that is excluded from coated pits into one that is integrated into coated pits and recycled with kinetics comparable to that of endocytic receptors (Lazarovits and Roth, 1988).

Lysosomal acid phosphatase (LAP) resembles other lysosomal membrane proteins in some of its features such as dense glycosylation of the ectoplasmic domain, short cytoplasmic tail of 19 residues, mannose 6-phosphate receptor independent targeting, and partial localization at the cell surface (Pohlmann *et al.*, 1988; Waheed *et al.*, 1988; Gottschalk *et al.*, 1989a). The pathway to lysosomes includes passage of the trans Golgi, cell surface and endosomes. After synthesis at the endoplasmic reticulum the precursors of LAP are transported to the trans Golgi with a $t_{1/2}$ of 30 min, from where they reach the cell surface within less than 10 min. The cell surface associated precursors are subject to rapid internalization and recycling to the cell surface. On average precursors are retained for 5–6 h in this cell surface/endosome pool and recycle more than 15 times between the cell surface and endosomes before they are delivered to lysosomes (Braun *et al.*, 1989). After transfer to lysosomes the precursors are subject to limited proteolysis, which generates the soluble mature form of LAP (Gottschalk *et al.*, 1989b).

In the present study we have analyzed the role of the cytoplasmic tail of LAP for targeting to lysosomes. We show that deletion of the cytoplasmic tail results in accumulation of the truncated LAP precursors at the cell surface. The truncated LAP precursors are transferred by a slow process ($t_{1/2}$ ~1 day) from the cell surface to lysosomes, where they are processed to mature forms. Substitution of the single tyrosine in the cytoplasmic tail by phenylalanine arrests transport at the cell surface to a similar extent as deletion of the entire cytoplasmic tail. Furthermore, the cytoplasmic tail of LAP is sufficient to direct non-lysosomal membrane proteins to lysosomes.

Results

The transmembrane domain of LAP is required for intracellular retention and the cytoplasmic tail for efficient targeting to lysosomes

The membrane associated LAP precursor, which represents the transport form of LAP, and the soluble LAP, which represents the mature form of LAP located in lysosomes, can be differentiated by size and their behavior in detergent condensation. In expressing baby hamster kidney (BHK) cells the 63 kd precursor is largely recovered in the detergent phase, whereas the 52 kd mature form is exclusively found in the aqueous phase (Figure 1).

Translation termination codons were introduced into the LAP cDNA before (LAP-RP2) and after (LAP-RP3) the membrane spanning domain (see Figure 2). LAP-RP2 was secreted into the medium (Figure 1) and exhibited acid phosphatase activity (data not shown). The small fraction of LAP-RP2 found intracellularly was recovered in the aqueous phase. Thus, the luminal domain of LAP forms a catalytically active protein, but lacks the structural features necessary for intracellular retention and targeting to lysosomes. LAP-RP3 encoded polypeptides were retained intracellularly and were processed to a soluble form indistinguishable from the mature form derived from wild type LAP (Figure 1). Subcellular fractionation by Percoll density centrifugation showed that the mature form of LAP-RP3 is enriched in dense lysosomes (Figure 3, upper panel). Compared to wild type LAP the transport of LAP-RP3 to dense lysosomes was significantly slower. While wild type LAP is transported to dense lysosomes with a $t_{1/2}$ of 6–7 h (Gottschalk *et al.*, 1989b), LAP-RP3 is transported with a $t_{1/2}$ of ~24 h to dense lysosomes (Figure 3, lower left panel). A further difference between wild type and LAP-RP3 concerns the proteolytic processing in dense lysosomes. Wild type LAP precursors persist for 6–7 h in dense lysosomes before they are processed to the mature form (Gottschalk *et al.*, 1989b). LAP-RP3 precursors were hardly detectable in dense lysosomes, suggesting that they are processed immediately after delivery to lysosomes. The slow transport of LAP-RP3 changes the equilibrium distribution of LAP activity between dense lysosomes and light membranes. In BHK cells expressing wild type LAP, 40% of total LAP enzyme activity (precursor and mature form) are associated with dense lysosomes (Waheed *et al.*, 1988; Gottschalk *et al.*, 1989b), while this fraction is diminished to 28% in BHK cells expressing LAP-RP3 (Figure 3, lower right panel).

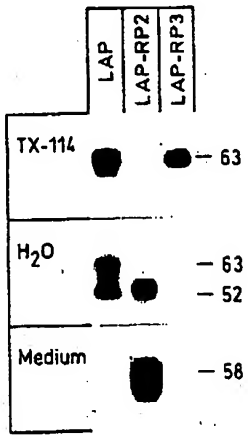

Fig. 1. Distribution of wild type and truncated forms of LAP in BHK cells. BHK cells expressing wild type LAP (LAP), LAP lacking the transmembrane domain and cytoplasmic tail (LAP-RP2) or LAP lacking the cytoplasmic tail (LAP-RP3) were metabolically labelled for 16 h with [^{35}S]methionine. The cell extract was phase separated with Triton X-114 into a detergent (TX-114) and an aqueous (H_2O) phase. The LAP was immunoprecipitated from the two phases and from the medium. The molecular weights of the membrane associated precursors (63 kd) and the mature forms (52 kd) of wild type LAP and of the secreted LAP-RP2 (58 kD) are indicated.

LAP-RP3 accumulates at the cell surface before transport to lysosomes

In BHK cells expressing wild type LAP between 15 and 20% of the LAP precursors are located at the cell surface. These precursors are in equilibrium with those in endosomes. In contrast to the intracellular precursors, the cell surface

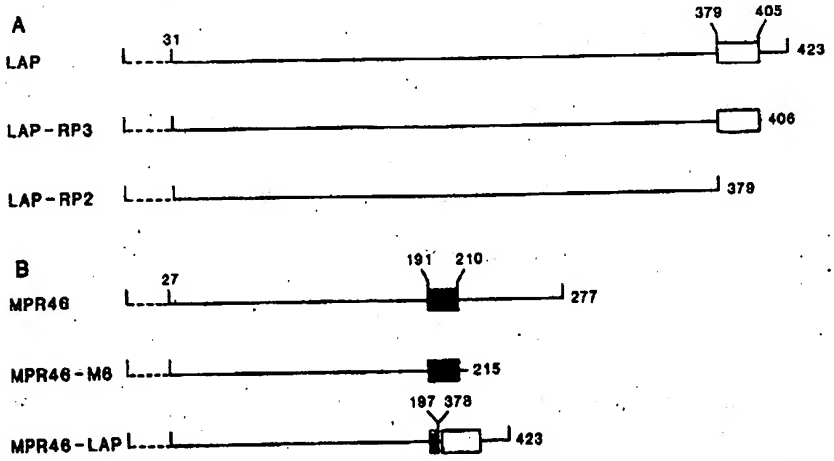

Fig. 2. Structure of the wild type and mutants of LAP, MPR46, and of the chimeric receptor-LAP polypeptides. N-terminal cleavable signal sequences are indicated by dashed lines. Boxes represent the transmembrane domain of LAP (open) and MPR46 (filled). The numbers refer to the first amino acid of the precursor with cleaved signal sequence, the membrane spanning domain, the cytoplasmic tail and to the C-terminal residue (for numbering of LAP and MPR46 see Pohlmann *et al.*, 1988 and 1987, respectively).

associated precursors are accessible at 4°C to antibodies, to neuraminidase or to lactoperoxidase catalyzed iodination (Braun *et al.*, 1989).

To determine the portion of cell surface-associated LAP, precursor cells were labeled for 2 h and chased for 2 h. Subsequently cells were incubated with neuraminidase at 4°C. The precursor LAP was immunoprecipitated from the detergent extract of cells and analyzed by SDS−PAGE or isoelectric focusing. Neuraminidase treatment decreases the size of the LAP precursors by about 3−4 kd and converts the acidic forms (pI 4.9−5.6) to more basic forms (pI 6.0−6.3) (Braun *et al.*, 1989). In BHK cells expressing wild type LAP, 15% of the precursors are accessible to neuraminidase as estimated by the shift of pI (Figure 4B). This fraction is too small to be detectable by SDS−PAGE (Figure 4A). In BHK cells expressing LAP-RP3 the pI of about 80% of the precursors was shifted to pI 6.0−6.3 (Figure 4B) and a reduction in size by 3−4 kd was apparent after separation by SDS−PAGE. This reduction in size was more obvious for the dimeric form of precursor LAP (Figure 4A). These results demonstrate that 2 h after synthesis about 80% of the LAP-RP3 precursors are located at the cell surface compared to 15% of wild type LAP.

To investigate the time span of accumulation of LAP-RP3 precursors at the cell surface, cells were labelled for 1 h, chased for 1−14 h and analyzed by SDS−PAGE (Figure 5). During the entire chase period essentially all LAP-RP3 precursors were accessibl to neuraminidase (desialylation decreases the size of the molecules by about 4 kd, see Figure 4A) and hence are located at the cell surface. In the same type of experiment performed with wild type expressing cells only about 10−13% of total LAP precursors were located at the cell surface. Furthermore, in contrast to wild type LAP (due to the slow transport f LAP-RP3 precursors to lysosomes) the total LAP signal hardly declined over the chase period of 14 h [compare with Braun *et al.* (1989) for wild type LAP data].

The arrest of the LAP-RP3 precursors at the cell surface was also demonstrated with the immunofluorescence approach of Lazarovits and Roth (1988). LAP antibodies were bound at 0°C to the cell surface of BHK cells and examined for their localization directly after binding and after

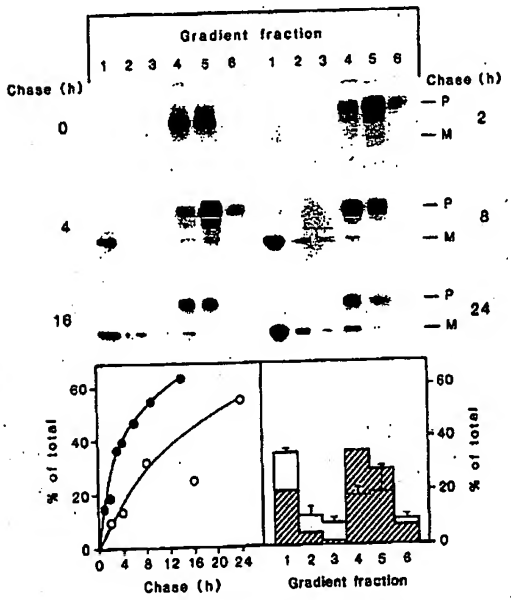

Fig. 3. Transport of LAP-RP3 mutant protein. Upper panel: LAP-RP3 expressing BHK cells were labeled for 1 h and chased for 0, 2, 4, 8, 16 or 24 h as indicated. The post-nuclear supernatants were subjected to Percoll density centrifugation. LAP-RP3 was immunoprecipitated from the gradient fractions and seperated by SDS−PAGE. P, LAP-RP3 precursor, membrane bound; M, LAP-RP3, mature, soluble form. Lower left panel: LAP polypeptides in dense lysosomes (gradient fractions 1 and 2) and light membranes (gradient fractions 4 and 5) were quantified by densitometry, and LAP in dense lysosomes expressed as percentage of total LAP (–o–). For comparison the data on transport of wild type LAP to lysosomes taken from Gottschalk *et al.* (1989b) are shown (–●–). *Lower right panel*: distribution of β-hexosaminidase (clear area) and acid phosphatase (hatched area) activities in gradient fractions 1−6 (from bottom to top). Bars indicate the variation of β-hexosaminidase activity in four individual gradients.

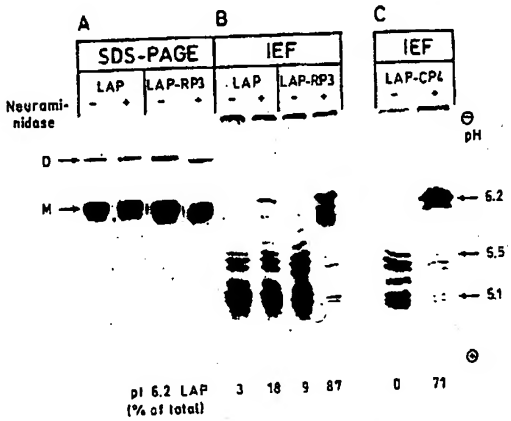

Fig. 4. Wild type LAP, LAP-RP3 and LAP-CP4 precursors accessible to neuraminidase at 4°C. Cells labeled and chased for 2 h at 37°C, respectively were incubated for 1 h at 4°C with or without neuraminidase. After phase separation of the cell extracts with Triton X-114, the precursor was immunoprecipitated from the detergent phase. Aliquots of the immunoprecipitates were subjected to SDS−PAGE (A) or isoelectric focussing (B, C). The numbers below the lanes represent the percentage of precursor shifted by neuraminidase to pH 6.0−6.3. M and D denote positions of LAP monomers and LAP dimers resistant to boiling in SDS and dithiothreitol, respectively.

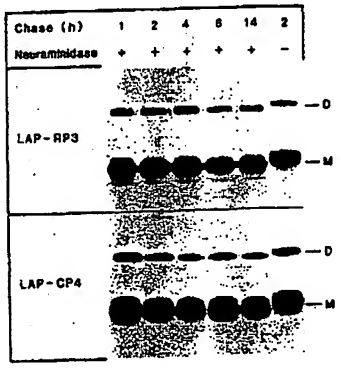

Fig. 5. Cell surface as the major localization of LAP-RP3 and LAP-CP4 precursors during a prolonged chase. BHK cells expressing LAP-RP3 or LAP-CP4 labeled for 1 h were chased for 1−14 h and then incubated at 4°C with neuraminidase as indicated above the lanes. After phase separation with Triton X-114 the precursors were immunoprecipitated and analyzed by SDS−PAGE. The positions of monomeric (M) and dimeric (D) forms are indicated.

reculturing of the cells for 60 min at 37°C. The punctuated pattern of antibodies bound at 4°C (Figure 6A,B) was replaced by a diffuse staining when the cells were fixed prior to incubation with LAP antibodies (not shown). It reflects therefore an antibody induced rather than a natural clustering of cell-surface associated LAP. After reculturing the cells at 37°C the antibodies bound to wild type LAP were rapidly internalized and accumulated in vacuoles (Figure 6A, bottom left), which distribute as the lysosomal marker arylsulfatase B (not shown) and hardly any antibodies were detectable at the cell surface (Figure 6B, bottom left). The traces of antibodies still present at th cell surface are attributed to the recycling of LAP (Braun *et al.*, 1989). In cells expressing LAP-RP3 the antibodies remained associated with the cell surface even after an incubation for 1 h at 37°C (Figure 6A,B, middle).

These results indicate that newly synthesized LAP-RP3 accumulates at the cell surface. From the cell surface LAP-RP3 precursors are transferred to dense lysosomes with a $t_{1/2}$ of ~1 day. Two independent observations suggest that

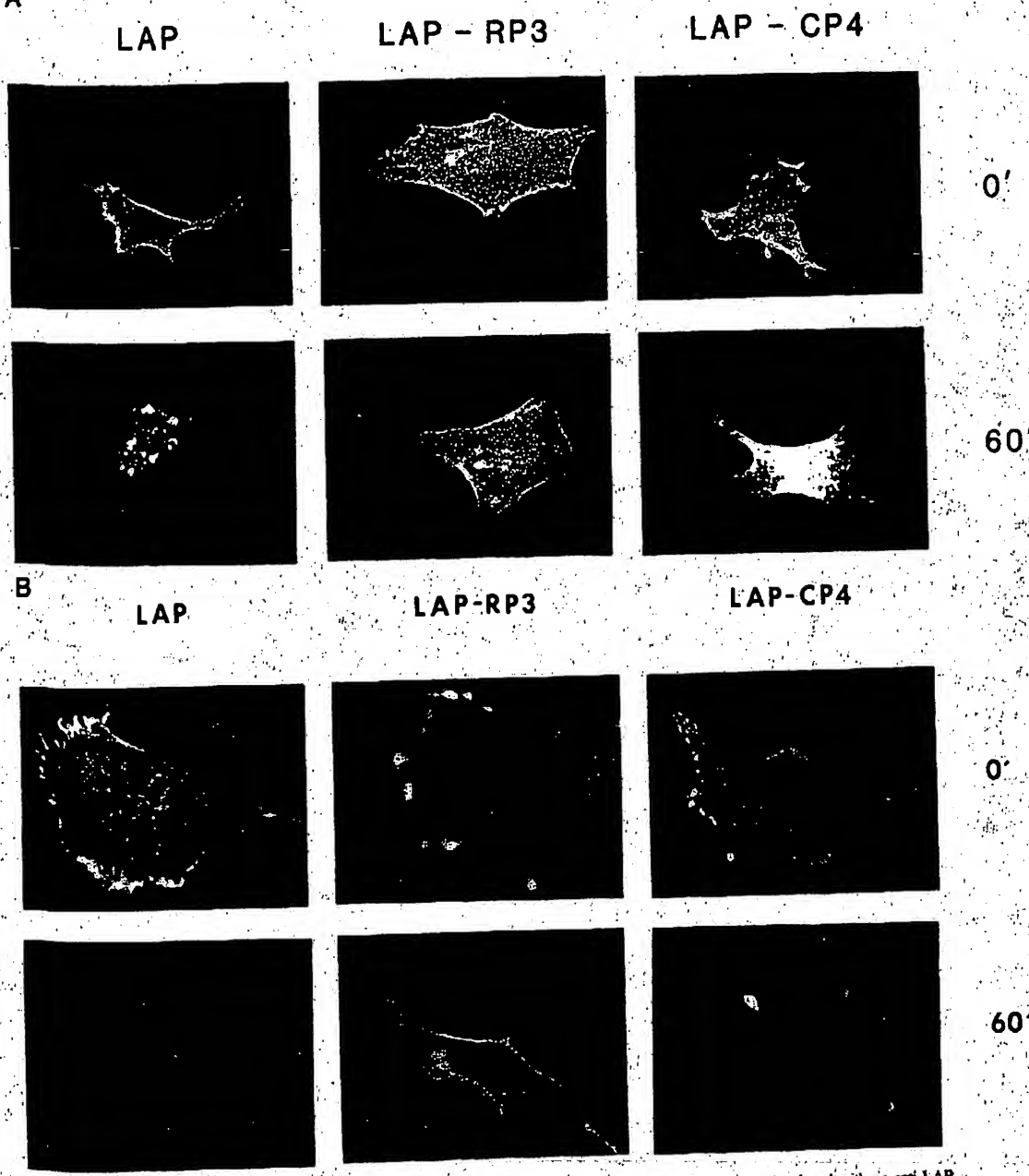

Fig. 6. Internalization of wildtype and mutant LAP. LAP, LAP-RP3 and LAP-CP4 expressing BHK cells were incubated with an anti-LAP antiserum for 1 h at 0°C. (A) Cells were washed and fixed immediately (0′) or incubated at 37°C for 60 min and then fixed with paraformaldehyde (60′). Cells were permeabilized with detergent and rhodamine conjugated immunoglobulin was used as second antibody. (B) For selective detection of cell surface associated antibodies cells were incubated with the second antibody (0′) or recultured at 37°C for 60 min and then incubated with the second antibody (60′) before fixation. Detergent permeabilization was omitted. The microscope was focused on the cell periphery of cells with surface staining and on vesicles when intracellular staining was observed.

LAP-RP3 precursors do not accumulate intracellularly during transport from the cell surface to lysosomes. Firstly, LAP-RP3 precursors are accessible to neuraminidase during prolonged chase (Figure 5), and secondly, LAP-RP3 precursors are not found in dense lysosomes (Figure 3).

Tyrosine 413 in the cytoplasmic tail is essential for rapid internalization

Tyrosine residues in the cytoplasmic tails of the LDL-receptor, a mutant hemagglutinin and the M_r 300 kd mannose 6-phosphate receptor are essential for rapid internalization of these polypeptides (Davis *et al.*, 1986, 1987; Lazarovits and Roth 1988; Lobel *et al.*, 1989). The cytoplasmic tail of LAP contains a single tyrosine in position 413. In LAP-CP4 this tyrosine was changed to phenylalanine (Figure 7). In LAP-CP4-expressing BHK cells, labeled for 2 h and chased for 2 h, 70% of the LAP-CP4 precursors were accessible to neuraminidase at 4°C (Figure 4C). During a chase for 1–14 h no intracellular LAP-CP4 precursors were detectable (Figure 5, lower panel). In the antibody internalization assay antibodies bound at 0°C to cells expressing LAP-CP4 remained cell-surface localized after reculturing for 1 h at 37°C as shown by the staining pattern of permeabilized (Figure 6A, bottom right) and non-permeabilized (Figure 6B, bottom right) cells. These results indicate that tyrosine 413 is an essential component of the signal in the cytoplasmic tail which is required for rapid internalization.

The cytoplasmic tail of LAP confers rapid internalization and lysosomal targeting to non-lysosomal proteins

The experiments outlined above had substantiated that the cytoplasmic domain is essential for rapid internalization of cell-surface located LAP precursors and thereby for its efficient transport to lysosomes, and that tyrosine 413 is an essential part of this endocytosis signal. To test whether this signal is not only essential but also sufficient for rapid internalization and lysosomal targeting a chimeric polypeptide consisting of the ectoplasmic and transmembrane domain of the plasma membrane sorted glycoprotein hemagglutinin (HA) and the cytoplasmic tail of LAP was constructed (HA–LAP, Figure 7B). In contrast to wild type HA the chimera HA–LAP is rapidly internalized since anti-HA antibodies bound to the cell surface at 0°C remained at the plasma membrane of wild type HA expressing cells whereas they were transferred to vesicular structures in HA–LAP expressing cells upon reculturing for 1 h at 37°C (Figure 8A, left and middle). Substitution of the single tyrosine residue in the cytoplasmic domain of the HA–LAP chimera (HA–CP4, Figure 7B) by phenylalanine reverted the rapid internalization almost completely (Figure 8A, right). The internalization (HA–LAP) or non-internalization (HA–CP4) of HA-associated antibodies during the reculturing period was mirrored by the disappearance or persistence of immunofluorescent staining at the cell surface, respectively, if the cells were not permeabilized prior to staining (Figure 8B).

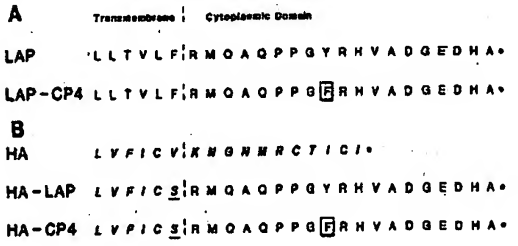

Fig. 7. Amino acid sequences of LAP mutants and HA–LAP chimeras. (A) In the mutant LAP-CP4 tyrosine 413 in the cytoplasmic domain of wild type (LAP) was changed to phenylalanine (boxed). (B) The ectoplasmic and transmembrane domains of influenza virus hemagglutinin (HA) were fused to the cytoplasmic domain of lysosomal acid phosphatase (HA–LAP). The C-terminal valine of the hemagglutinin (HA) transmembrane domain was changed to serine (underlined) due to the introduction of an *Xhol* restriction site (see Materials and methods). For construction of HA–CP4 the tyrosine 413 of the HA–LAP cytoplasmic domain was changed to phenylalanine (boxed). Sequences are shown in one letter code.

As a second indicator molecule we utilized the M_r 46 kd mannose 6-phosphate receptor (MPR46). A chimeric protein consisting of the ectoplasmic domain and 6 amino acids of the transmembrane domain of MPR46 and the complete transmembrane and cytoplasmic domains of LAP was constructed (MPR46–LAP; see Figure 2B and Materials and methods). Wild type MPR46 recycles between Golgi apparatus and endosomes and between plasma membrane and endosomes; it does not enter lysosomes in detectable amounts (Stein *et al.*, 1987a; Bleekemolen *et al.*, 1989). An endocytosis signal necessary for rapid internalization is residing in the cytoplasmic tail of the receptor (B.Weber and R.Pohlmann, unpublished results). This is also shown in the antibody internalization assay depicted in Figure 9; receptor antibodies bound to the cell surface of BHK cells expressing wild type MPR46 are endocytosed into small vesicular structures most likely representing endosomes within 1 h of reculturing at 37°C. In contrast receptor antibodies bound to truncated MPR46 lacking all but 6 amino acids of the cytoplasmic domain (MPR46-M6; Figure 2B) remain at the plasma membrane (Figure 9A and B, left and middle). Receptor antibodies bound to the cell surface of BHK cells expressing the MPR46–LAP chimera were readily internalized into larger lysosome-like vesicles (Figure 9A, right) and cleared from the plasma membrane after 1 h of reculturing at 37°C (Figure 9B, right). Since the transmembrane domain of LAP is not sufficient for rapid internalization of LAP, rapid endocytosis of the MPR46–LAP chimera is most likely due to the cytoplasmic tail of LAP.

The half life of MPR46–LAP polypeptides is approximately 2 h, as determined by metabolic labeling of expressing BHK cells for 1 h, and chasing for appropriate times, followed by immunoprecipitation and quantification of MPR46–LAP polypeptides (data not shown). Despite this short half life MPR46–LAP fusion protein must be considered folded correctly since it binds to phosphomannan in a mannose 6-phosphate dependent manner (B.Weber and R.Pohlmann, unpublished) and undergoes carbohydrate processing as wild type MPR46. Furthermore comparable amounts of wild type MPR46 (17%) and MPR46-LAP chimera (18%) are expressed at the cell surface as detected by [^{125}I] antibody binding. 45% of cell surface bound [^{125}I] MPR46 antibodies were internalized within 3 min upon warming to 37°C in cells expressing MPR46–LAP, as compared with 51% in wild type MPR46-expressing cells. If the MPR46–LAP chimera recycles between endosomes and cell surface as do wild type MPR46 and LAP it probably exits from the recycling pool much faster and is delivered

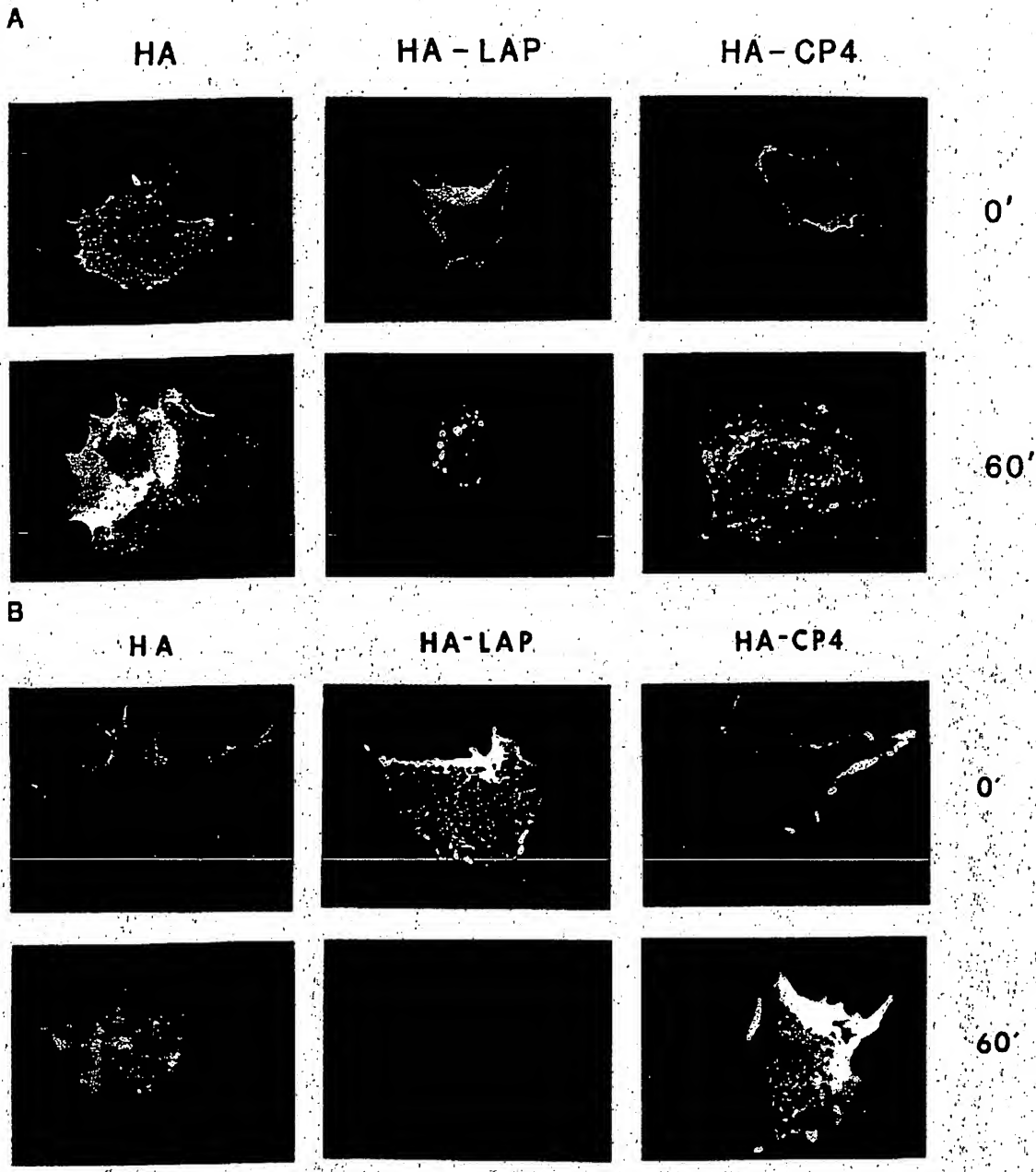

Fig. 8. Internalization of hemagglutinin (HA) and hemagglutinin-LAP fusion proteins (HA–LAP, HA–CP4). HA, HA–LAP and HA–CP4 expressing BHK cells were incubated with an anti-HA antiserum for 1 h at 0°C. Subsequently cells were processed for indirect immunofluorescence as described in Figure 6 legend. (A) with detergent permeabilization, (B) without detergent permeabilization.

to lysosomes. In contrast to the ectoplasmic domain of LAP, that of MPR46 is likely to be sensitive to lysosomal proteinases since MPR46 does not enter this compartment. To test this hypothesis lysosomal proteolysis was inhibited by preincubation of MPR46–LAP and wild type MPR46 expressing cells with lysosomal proteinase inhibitors leupeptin or/and pepstatin. Metabolic labeling for 2 h and subsequent chase for 6 h, followed by immunoprecipitation of MPR46-related polypeptides, showed that the chimera was stabilized in the presence of these inhibitors whereas no detectable effect on the yield of wild type MPR46 was observed (Figure 10).

A Percoll density gradient centrifugation of post nuclear supernatant from BHK cells metabolically pulse–chase labeled for 2 h each under the proteolytic protection of leupeptin and pepstatin, produced direct evidence of delivery of the MPR46–LAP chimera to dense lysosomes (Figure 11, bottom panel). More than 50% of immunoprecipitable MPR46–LAP were associated with heavy membranes whereas wild type MPR46 and truncated receptors lacking the cytoplasmic domain (MPR46-M6) were absent from dense lysosomes and associated with fractions of low density (Figure 11, top and middle panel), which are enriched in markers for the endoplasmic reticulum, Golgi, plasma

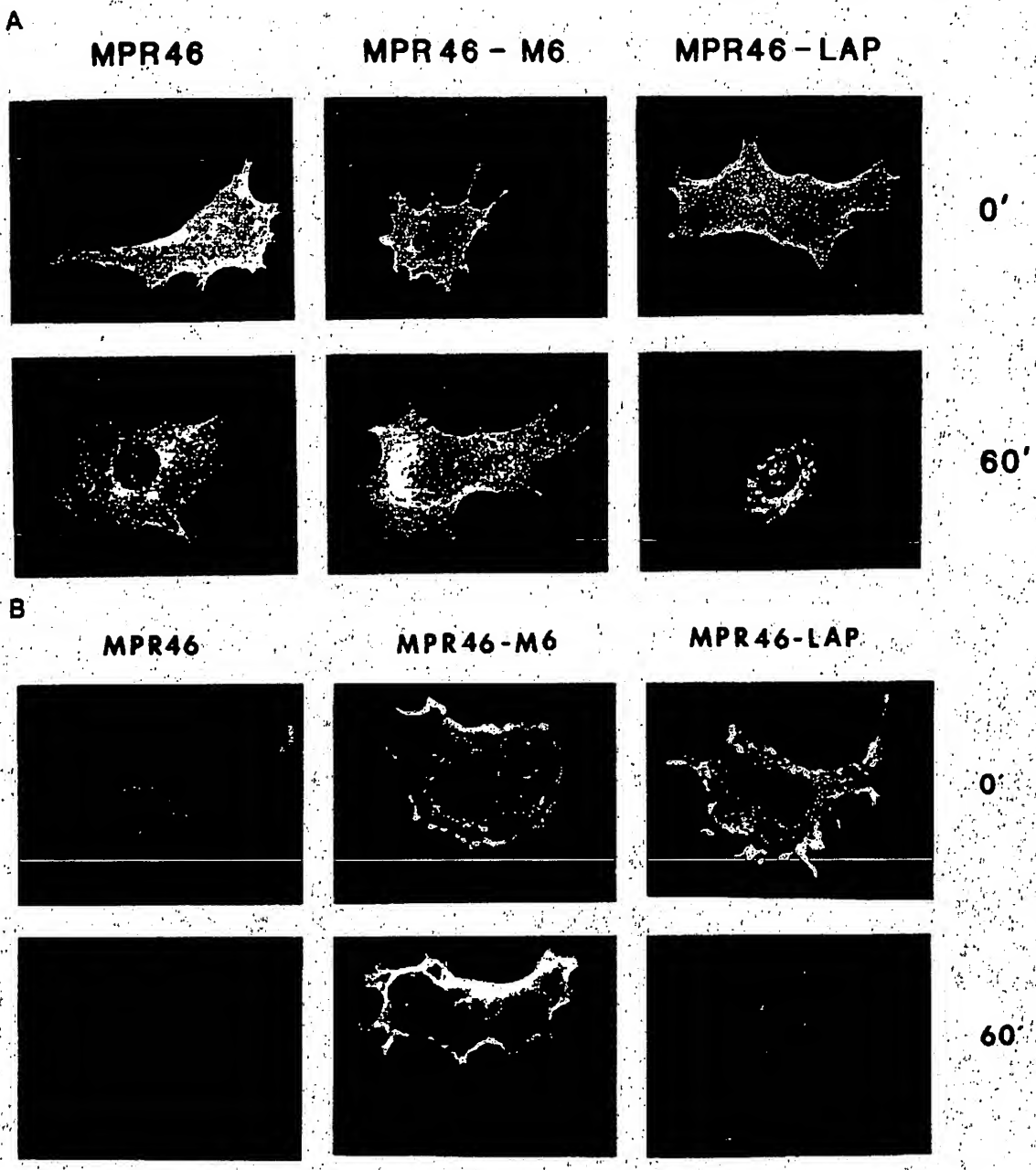

Fig. 9. Internalization of MPR46, MPR46–M6 and MPR46–LAP. MPR46, MPR46–M6 and MPR46–LAP expressing BHK cells were incubated with an anti-MPR46 antiserum for 1 h at 0°C. Subsequently cells were processed for indirect immunofluorescence as described in Figure 6 legend. (A) with detergent permeabilization, (B) without detergent permeabilization.

membrane and endosomes (Lemansky *et al.*, 1984). These observations show that the MPR46–LAP chimeras are delivered to lysosomes, while the wild type MPR46, as well as receptors lacking a cytoplasmic domain, are excluded from lysosomes.

Discussion

Transport of LAP precursor lacking the cytoplasmic tail

A tyrosine-containing endocytosis signal in the cytoplasmic tail is required for targeting of LAP to lysosomes. LAP precursors are associated for about 5–6 h with a cell surface/endosome pool before they are delivered to lysosomes. Within these 5–6 h they recycle more than 15 times between the cell surface and endosomes. At steady state about 4 times more LAP precursors are present in endosomes than at the cell surface (Braun *et al.*, 1989). The present study shows that the recycling between the cell surface and endosomes is interrupted if the cytoplasmic tail of LAP is deleted, or tyrosine 413 in the cytoplasmic tail is changed to phenylalanine. The mutant LAP precursors accumulate at the cell surface. This indicates that the cytoplasmic tail contains a signal required for internalization

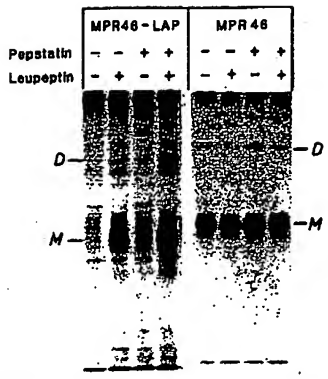

Fig. 10. Expression of chimeric receptor–LAP polypeptide (MPR46–LAP) in comparison to wild type receptor (MPR46). BHK cells expressing MPR46 or MPR46–LAP were incubated with 100 μM of proteinase inhibitors pepstatin and leupeptin as indicated for 16 h prior to labeling. Cells were labeled for 2 h and chased for 6 h in the presence of the same inhibitors. Receptor related polypeptides were isolated by immunoprecipitation and analyzed by SDS–PAGE. M and D denote monomeric and dimeric receptor polypeptides, respectively.

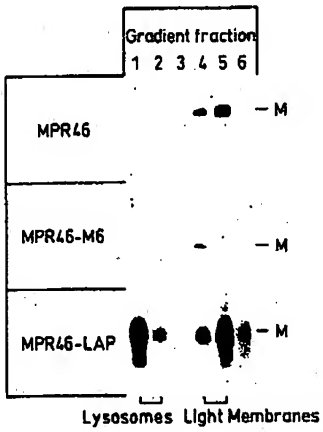

Fig. 11. Subcellular distribution of wild type receptor (MPR46), truncated receptor (MPR46–M6) and chimeric receptor–LAP polypeptide MPR46–LAP. BHK cells expressing MPR46, MPR46–M6 or MPR46–LAP were incubated with 100 μM of proteinase inhibitors pepstatin and leupeptin for 16 h and pulse labeled and chased for 2 h each. Post nuclear supernatants were subjected to subcellular fractionation by Percoll gradient centrifugation. Receptor related polypeptides were isolated by immunoprecipitation and analyzed by SDS–PAGE. Dense lysosomes migrate in fractions 1 and 2 and light membranes in fractions 4 and 5 as indicated. M, monomeric receptor polypeptides.

of LAP and that tyrosine 413 is an essential part of this signal.

The importance of tyrosine-containing signals within the cytoplasmic tail for rapid internalization of cell surface associated proteins has been reported for the LDL-receptor (Davis *et al.*, 1986, 1987), an artificially created mutant of the influenza virus hemagglutinin (Lazarovits and Roth, 1988) and the 300 kd mannose 6-phosphate/IGF II receptor (Lobel *et al.*, 1989). In the LDL receptor phenylalanine and tryptophan can substitute for the tyrosine, while a change to charged or uncharged aliphatic residues results in a loss of internalization. Nonsense mutations following immediately the tyrosine in the LDL receptor also disrupt internalization, suggesting that the endocytosis signal comprises more than the tyrosine residue (Davis *et al.*, 1987). The requirement for a suitable sequence around the tyrosine is supported by the studies of Lazarovits and Roth (1988). They showed that changing residues in the short cytoplasmic tail of HA to tyrosine caused rapid internalization of HA only in one of three positions tested. Comparison of the sequence around the tyrosines in recycling receptors show no apparent sequence homologies, suggesting that a variety of combinations of neighboring residues can provide the appropriate surrounding for the tyrosine. Moreover, the capability of phenylalanine to substitute for tyrosine in the LDL tail (Davis *et al.*, 1987) but not in the tail of the mutant HA (Lazarovits and Roth, 1988) and of LAP suggests that neighboring residues determine whether phenylalanine is sufficient or tyrosine is required.

Cell surface proteins, which are rapidly internalized, cluster in clathrin-coated pits before endocytosis via clathrin-coated vesicles. Clustering in coated pits is mediated by the protein adaptor complex HAII, which recognizes the endocytosis signal in the cytoplasmic tail of cell surface proteins and clathrin (Pearse, 1988; Glickman *et al.*, 1989; Ahle and Ungewickell, 1989).

LAP precursors lacking the endocytosis signal are not permanently arrested at the cell surface. The first mutant LAP molecules are detectable in dense lysosomes 2 h after biosynthesis. About 1 day is required to deliver half of the precursors to dense lysosomes. We suppose that the LAP precursors lacking the endocytosis signal are delivered to lysosomes by a non-selective, stochastic process. It is not clear whether the mutant LAP precursors recycle to the cell surface as do wild type precursors. This would be expected if recycling depends on signals in the luminal and/or transmembrane domain of LAP. Due to the slow rate of internalization the endosomal pool of the mutant LAP precursors is expected to be much smaller and may therefore escape detection. The half life of an average cell surface polypeptide in fibroblasts is 20 h (Draye *et al.*, 1988). Cell surface polypeptides are usually degraded in lysosomes within less than 1 h. Therefore, their half lives are determined primarily by the transport rate to lysosomes (Draye *et al.*, 1988). The similarity of the transport rates of the mutant LAP precursor and average cell surface polypeptides to lysosomes suggests that they are transported to lysosomes by a common mechanism, which does not depend on specific signals. In contrast to average cell surface polypeptides, the mutant LAP precursors are not degraded in lysosomes but processed to soluble forms, which are indistinguishable from the mature form of wild type LAP.

The cytoplasmic tail inhibits proteolytic processing of LAP

The proteolytic processing of the LAP precursor after delivery to lysosomes is initiated by a cleavage within the cytoplasmic tail. This reaction is catalyzed by a thiol proteinase and occurs with a half time of 6–7 h. Only the product of the thiol proteinase serves as a substrate for an intralysosomal aspartyl proteinase, which rapidly generates the soluble mature form of LAP (Gottschalk *et al.*, 1989b). The rapid processing of truncated LAP after delivery t lysosomes supports the view that the presence of the

cytoplasmic tail delays generation of soluble LAP and that inhibition of premature proteolytic processing may be one f the tail functions.

The cytoplasmic tail of LAP is sufficient to target non-lysosomal proteins to lysosomes

The cytoplasmic tail of LAP was sufficient to induce rapid internalization of HA and MPR46. Internalization of the HA chimera was dependent on tyrosine 413 in the cytoplasmic LAP tail. When lysosomal proteolysis was inhibited, transport of the MPR46–LAP chimera to lysosomes could be demonstrated. The MPR46-LAP chimera contained the transmembrane domain and cytoplasmic tail of LAP. Since truncated forms of MPR46 and LAP containing their own transmembrane domains, but lacking their cytoplasmic tails, are not targeted to lysosomes, transport of the MPR46–LAP chimera to lysosomes can be ascribed to signals in the cytoplasmic tail provided by LAP. The fusion between MPR46 and LAP was not precisely at the border between the ectoplasmic domain of MPR46 and the transmembrane domain of LAP (the chimera contained the 6 initial residues of the transmembrane domain of MPR46). We therefore had to rule out the possibility that due to aberrant folding of the ectoplasmic domain the chimera was transported to lysosomes by default. The chimera binds mannose 6-phosphate, it forms dimers and its *N*-linked oligosaccharides are processed to complex type structures. Generation of a ligand binding site, dimerization and oligosaccharide processing involve a series of post-translational modifications of the ectoplasmic domain (Hille *et al.*, 1990). In addition, the chimera was transported to the cell surface, where it mediated the endocytosis of receptor antibodies. These data indicate that the ectoplasmic domain of the chimera is folded correctly.

The chimeric receptor had a half life of ≥ 2 h, indicating that it is transported to lysosomes much faster than wild type LAP. Transport of LAP to lysosomes is delayed by efficient recycling to the cell surface. The inability of the transmembrane domain and the cytoplasmic tail of LAP to induce a similar recycling of the chimeric receptor suggests that the structural requirements for recycling of LAP are provided by the ectoplasmic domain of LAP.

Targeting of other lysosomal membrane proteins

For several proteins of the lysosomal membrane the primary structure is known (Howe *et al.*, 1988; Viitala *et al.*, 1988; Fukuda *et al.*, 1989; Noguchi *et al.*, 1989). They all have a short carboxy-terminal cytoplasmic tail, which contains a single tyrosine residue. In a related study Mathews and Fambrough (1988) observed that the transmembrane and cytoplasmic domains of the lysosomal membrane protein LEP-100 are sufficient to target the ectoplasmic domain of the VSVG-protein, a plasma membrane protein, to lysosomes, while the opposite chimera containing the ectoplasmic domain of LEP-100 and the transmembrane domain and cytoplasmic tail of the VSVG-protein accumulates at the cell surface. They concluded that the transmembrane and cytoplasmic domains of LEP-100 contain the signals required for targeting to lysosomes.

It is therefore conceivable that passage of the cell surface is obligatory for many lysosomal glycoproteins and that a tyrosine containing endocytosis signal is essential for their efficient routing from the cell surface to lysosomes.

Materials and methods

Recombinant DNAs

The coding region of influenza virus HA from fowl plague virus, strain A/FPV/Rostock/34(H7N1) contained in plasmid pUcHA651 (Kuroda *et al.*, 1986) was subcloned into M13mp11 and kindly provided by E. Kretschmer and H.-D.Klenk, Marburg, FRG. The cDNAs for LAP (Pohlmann *et al.*, 1988) and MPR46 (Pohlmann *et al.*, 1987) were subcloned into M13mp18. For *in vitro* mutagenesis the procedure of Nakamaye and Eckstein (1986) was used. The codons for the LAP residues 380 and 407 were changed to amber codons (LAP-RP2 and LAP-RP3). The codon of MPR46 residue 216 was changed to an opal codon (MPR46-M6), the codon of LAP residue tyrosine 413 was changed to the phenylalanine codon TTC (Figure 7A). For construction of HA–LAP chimeras (HA–LAP and HA–CP4) a *Xho*I restriction site was introduced into the LAP cDNA at nucleotide 1209 and a *Xho*I restriction site into hemagglutinin DNA at nucleotide 1674. The HA DNA encoding the N-terminal part of the protein and the LAP cDNA encoding the C-terminal part of the protein were fused at these artificial *Xho*I restriction sites. This manipulation changed valine 552, the last amino acid residue of the HA transmembrane domain, to serine (underlined in Figure 7B). The codon for tyrosine 413 of HA–LAP was changed to the phenylalanine codon TTC yielding the chimera HA–CP4 (Figure 7B). For construction of the MPR46–LAP chimera a *Pst*I site was introduced at position 587 of the MPR46 cDNA. The MPR46 cDNA encoding the N-terminal part of the receptor was fused at this artificial *Pst*I site to the unique *Pst*I site of the LAP cDNA fragment encoding the C-terminal part of the protein. The construct encodes for the N-terminal 197 amino acids of MPR46 (with exchange of phenylalanine 196 to serine), followed by the transmembrane and cytoplasmic domain of LAP (see Figure 2).

The cDNAs encoding LAP mutants were subcloned into the *Eco*RI site of the expression vector pBEH (Artelt *et al.*, 1988). The *Bgl*II–*Bgl*II DNA fragment encoding HA was subcloned into the *Bam*HI site of plasmid pBEH. DNAs encoding the chimeras HA–LAP and HA–CP4 were subcloned as *Bgl*II–*Hin*dIII DNA fragments into *Bam*HI and *Hin*dIII restriction sites of the same expression vector. The MPR46 mutants and the MPR46–LAP chimera were subcloned as *Bgl*II–*Eco*RI inserts into *Bgl*II/*Eco*RI cut pBHE-P29Δ5′. In the pBHE-P29Δ5′ the 5′-untranslated sequence of the MPR46 cDNA is shortened to 7 bp to improve expression (Wendland *et al.*, 1989). All mutations were confirmed by sequencing of single and double stranded DNA.

Transfection of BHK cells

Baby hamster kidney cells (clone BHK-21), 5×10^5 cells/6 cm dish, were transfected with 10–20 μg of plasmid DNA, 0.5–1 μg pSV2pac (Vara *et al.*, 1986) using the calcium phosphate technique (Wigler *et al.*, 1977). No glycerol shock was applied. Two days after transfection the medium was supplemented with 2 μg/ml puromycin and 6 days later with 5 μg/ml puromycin. Alternatively 0.5–1 μg pSV2neo (Southern and Berg, 1982) were included in calcium phosphate precipitates, additionally, medium was supplemented with 2 μg/ml puromycin two days after transfection and with 30 μg/ml puromycin and 500 μg/ml G418 six days later. Single colonies were picked and either analyzed for L-tartrate inhibitable LAP activity (Waheed *et al.*, 1985) or synthesis of MPR46 or hemagglutinin related proteins.

Metabolic labeling and preparation of cell extracts

BHK cells in 35 or 60 mm dishes were labeled with 0.74–11.1 MBq [^{35}S]methionine ($\geq$24.6 TBq/mmol) as described (von Figura *et al.*, 1983; Lemansky *et al.*, 1985). During chase, the medium was supplemented with 0.25 mg/ml methionine. The cells were harvested by scraping and extracts of cells and media were prepared as described (Lemansky *et al.*, 1985). In some experiments soluble and membrane-associated cellular proteins were separated by Triton X-114 condensation (Stein *et al.*, 1987b).

Subcellular fractionation in Percoll gradients, immunoprecipitation, neuraminidase treatment and electrophoresis were performed essentially as described (Braun *et al.*, 1989; Wendland *et al.*, 1989).

Determination of cell surface expression and internalization of MPR46–LAP chimera and wild type MPR46

The monoclonal antibody 21D3 (A. Hille, unpublished) against the luminal domain of human MPR46 was iodinated as described (Parker and Strominger, 1983). Surface associated and total cellular wild type MPR46 and MPR46–LAP chimera were determined essentially as described (Wendland *et al.*, 1990).

For determination of antibody internalization, confluent 35 mm dishes of BHK cells expressing MPR46–LAP chimera or wild type MPR46 were incubated for 3×5 min at 4°C with medium (MEM containing 20 mM HEPES, pH 7.2 and 7.5% FCS), followed by an incubation for 2 h at 4°C

with [^{125}I] 21D3 antibody (360 000 c.p.m. in 0.6 ml medium). Subsequently cells were washed 3 times with medium at 4°C and either recultured for 3 min at 37°C or kept at 4°C. After incubation in medium at 4°C for another 5 min cells were washed 3 times with 0.6 ml 25 mM glycine/HCl, pH 2.5, 150 mM NaCl at 4°C for 8 min each. The cells were harvested by scraping in 2×0.5 ml 1 M NaOH. Radioactivity released by acidic washes (cell surface bound antibodies) and cell bound radioactivity (internalized antibodies) was determined.

Endocytosis assay

BHK cells expressing LAP, MPR46 or HA-related polypeptides were washed 3× with ice cold PBS and incubated for 1 h at 4°C with antisera diluted 1:150–1:300 in PBS. Subsequently cells were washed 3× with medium supplemented with 7.5% fetal calf serum to remove unbound antibodies and either fixed directly or incubated for 60 min at 37°C, 5% CO_2 and then fixed. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100. The cells were then incubated with second antibodies (goat anti-rabbit immunoglobulin or rabbit anti-goat immunoglobulin) conjugated to rhodamine. Alternatively the cells—either with or without reculturing at 37°C for 60 min— were incubated with the second antibody at 4°C and then fixed. This permitted the selective detection of cell surface associated antibodies.

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References

Ahle,S. and Ungewickell,E. (1989) *J. Biol. Chem.*, 264, 20089–20093.
Arnelt,P., Morelle,C., Ausmeier,M., Fitzek,M. and Hauser,H. (1988) *Gene*, 68, 213–219.
Barriocanal,J.G., Bonifacino,J.S., Yuan,L. and Sandoval,I.V. (1986) *J. Biol. Chem.*, 261, 16755–16763.
Bleekemolen,J.E., Stein,M., von Figura,K., Slot,J.W. and Geuze,H.J. (1989) *Eur. J. Cell Biol.*, 47, 366–370.
Braun,M., Waheed,A., von Figura,K. (1989) *EMBO J.*, 8, 3633–3640.
Chen,J.W., Cha,Y., Yuksel,K.U., Gracy,R.W. and August,J.T. (1988) *J. Biol. Chem.*, 263, 8754–8758.
Chen,J.W., Murphy,T.L., Willingham,M.C., Pastan,I., and August,J.T. (1985) *J. Cell Biol.*, 101, 85–89.
Davis,C.G., Lehmann,M.A., Russell,D.W., Anderson,R.G.W., Brown,M.S. and Goldstein,J.L. (1986) *Cell*, 45, 15–24.
Davis,G.G., van Driel,I.R., Russell,D.W., Brown,M.S. and Goldstein,J.L. (1987) *J. Biol. Chem.*, 262, 4075–4082.
Draye,J.P., Courtoy,P.J. Quintart,J. and Baudhuin,P. (1988) *J. Cell Biol.*, 107, 2109–2115.
Farnbrough,D.M., Takeyasu,K., Lippincott-Schwartz,J., Siegel,N.R., and Somerville,D. (1988) *J. Cell Biol.*, 106, 61–67.
Fukuda,M., Viitala,J., Matteson,J. and Carlsson,S.R. (1989) *J. Biol. Chem.*, 263, 18920–18928.
Glickman,J.N., Couibear,E. and Pearse,B.W.F. (1989) *EMBO J.*, 8, 1041–1047.
Gottschalk,S., Waheed,A. and von Figura,K. (1989a) *Biol. Chem. Hoppe-Seyler*, 370, 75–80.
Gottschalk,S., Waheed,A., Schmidt,B., Laidler,P. and von Figura,K. (1989b) *EMBO J.*, 8, 3215–3219.
Hille,A., Waheed,A. and von Figura,K. (1990) *J. Cell Biol.*, 110, 963–972.
Howe,C.L., Granger,B.L., Hull,M., Green,S.A., Gabel,C.A., Helenius,A. and Mellman,I. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 7577–7581.
Kuroda,K., Hauser,C., Rott,R., Klenk,H.-D. and Doerfler,W. (1986) *EMBO J.*, 5, 1359–1365.
Jacopetta,B.J., Rothenberger,S. and Kühn,L.C. (1988) *Cell*, 54, 485–489.
Lazarovits,J. and Roth,M. (1988) *Cell*, 53, 743–752.
Lemansky,P., Gieselmann,V., Hasilik,A. and von Figura,K. (1984) *J. Biol. Chem.*, 259, 10129–10135.
Lemansky,P., Gieselmann,V., Hasilik,A. and von Figura,K. (1985) *J. Biol. Chem.*, 260, 9023–9030.
Lewis,V., Green,S.A., Marsh,M., Vihko,P, Helenius,A. and Mellman,I. (1985) *J. Cell Biol.*, 100, 1839–1847.
Lippincott-Schwartz,J. and Farnbrough,D.M. (1987) *Cell*, 49, 669–677.
Lippincott-Schwartz,J. and Farnbrough,D.M. (1986) *J. Cell Biol.*, 102, 1593–1605.
Lobel,P., Fujimoto,K., Ye,R.D., Griffiths,G. and Kornfeld,S. (1989) *Cell*, 57, 787–796.
Mathews,P.M. and Farnbrough,D.M. (1988) *J. Cell Biol.*, 107, 439a.
Mostov,K.E., de Bruyn Kops,A. and Deitcher,D.L. (1986) *Cell*, 47, 359–364.
Nakamaye,K.L. and Eckstein,F. (1986) *Nucleic Acids Res.*, 14, 9679–9698.
Noguchi,Y., Himeno,M., Sasaki,H., Tanaka,Y., Kono,A., Sakaki,Y., and Kato,K. (1989) *Biochem. Biophys. Res. Commun.*, 164, 1113–1120.
Parker,K.C. and Strominger,J.C. (1983) *Biochemistry*, 22, 1145–1153.
Pearse,B.M.F. (1988) *EMBO J.*, 7, 3331–3336.
Pohlmann,R., Krentler,C., Schmidt,B., Schröder,W., Lorkowski,G., Cully,J., Mersmann,G., Geier,C., Waheed,A., Gottschalk,S., Grzeschik,H., Hasilik,A. and von Figura,K. (1988) *EMBO J.*, 7, 2343–2350.
Pohlmann,R., Nagel,G., Schmidt,B., Stein,M., Lorkowski,G., Krentler,C., Cully,J., Meyer,H.E., Grzeschik,K.H., Mersmann,G., Hasilik,A. and von Figura,K. (1987) *Proc. Natl. Acad. Sci. USA*, 84, 5575–5579.
Prywes,R., Livuek,E., Ullrich,A. and Schlessinger,J. (1986) *EMBO J.*, 5, 2179–2190.
Rothenberger,S., Jacopetta,B.J. and Kühn,L.C. (1987) *Cell*, 49, 423–431.
Stein,M., Zijderhand-Bleekemolen,J.E., Geuze,H.J., Hasilik,A. and von Figura,K. (1987a) *EMBO J.*, 6, 2677–2681.
Stein,M., Braulke,T., Krentler,C., Hasilik,A. and von Figura,K. (1987b) *Biol. Chem. Hoppe-Seyler*, 368, 937–947.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, 1, 327–341.
Vara,J.A., Portela,A., Ortin,J. and Jiminez,A. (1986) *Nucleic Acids Res.*, 14, 4617–4624.
Viitala,J., Carlsson,S.R., Siebert,P.D. and Fukuda, M. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 3743–3747.
von Figura,K., Steckel,F. and Hasilik,A. (1983) *Proc. Natl. Acad. Sci. USA*, 80, 6066–6070.
Waheed,A. and van Etten,R. (1985) *Arch. Biochem. Biophys.*, 243, 274–283.
Waheed,A., Gottschalk,S., Hille,A., Krentler,C., Pohlmann,R., Braulke,T., Hauser,H., Geuze,H. and von Figura,K. (1988) *EMBO J.*, 7, 2351–2358.
Wendland,M., Hille,A., Nagel,G., Waheed,A., von Figura,K. and Pohlmann,R. (1989) *Biochem. J.*, 260, 201–206.
Wendland,M., Waheed,A., von Figura,K. and Pohlmann,R. (1990) submitted for publication.
Wigler,M., Silverstein,S., Lee,L.S., Pellicer,A., Cheng,T. and Axel,R. (1977) *Cell*, 11, 223–232.

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Multiple mRNAs encode the avian lysosomal membrane prot in LAMP-2, resulting in alternative transmembrane and cytoplasmic domains

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SUMMARY

Lysosomal membranes are enriched in extensively glycosylated transmembrane proteins, LAMP-1 and LAMP-2. LAMP-1 proteins have been characterized from several mammalian species and from chickens, but no non-mammalian homologues of LAMP-2 have been described, and no splice variants of either protein have been reported. Here we report the characterization of three cDNA clones encoding chicken LAMP-2. The nucleotide sequences of the cDNAs diverge at their 3′ ends within the open reading frame, resulting in sequences that code for three different transmembrane and cytoplasmic domains. Southern analysis suggests that a single gene encodes the common region of chicken LAMP-2. The position of the divergence and the identity of the common sequence are consistent with alternative splicing of 3′ exons. Analysis of the mRNAs present in adult chicken tissues suggests tissue-specific expression of the three chicken LAMP-2 variants, with LAMP-2b expressed primarily in the brain. The cytoplasmic domain of LAMP-type proteins contains the targeting signal for directing these molecules to the lysosome. Using chimeras consisting of the lumenal domain of chicken LEP100 (a LAMP-1) and the transmembrane and cytoplasmic domains of the LAMP-2 variants, we demonstrate in transfected mouse L cells that all three LAMP-2 carboxyl-terminal regions are capable of targeting the chimeric proteins to lysosomes. Levels of expression, subcellular distribution, and glycosylation of the LAMP proteins have all been shown to change with differentiation in mammalian cells and to be correlated with metastatic potential in certain tumor cell lines. Alternative splicing of the LAMP-2 transcript may play a role in these changes.

Key words: lysosome, chicken, protein targeting, alternative splicing

INTRODUCTION

Lysosomal membranes are enriched in extensively glycosylated proteins (Kornfeld and Mellman, 1989) that can be divided into several classes based on sequence homology and structural similarity (Fukuda, 1991). cDNAs encoding members of the LAMP-1 and LAMP-2 classes have been characterized from several species, including LAMP-1s from human (Viitala et al., 1988; Fukuda et al., 1988), rat (Howe et al., 1988; Himeno et al., 1989), mouse (Chen et al., 1988) and chicken (Fambrough et al., 1988), and LAMP-2s from human (Fukuda et al., 1988), mouse (Cha et al., 1990; Granger et al., 1990) and rat (Noguchi et al., 1989; Granger et al., 1990). On the basis of their deduced amino acid sequences and protein biochemistry, proteins of the LAMP-1 and LAMP-2 types are evolutionarily related and structurally similar. They contain a large lumenal domain which is heavily glycosylated, a single transmembrane domain, and a short cytoplasmic domain. A glycine-tyrosine motif present in the cytoplasmic domains of LAMP-1 and LAMP-2 is involved in targeting these proteins to the lysosome (Williams and Fukuda, 1990; Hunziker et al., 1991; Guarnieri et al., 1993). Consistent with phylogenetic relatedness, the genes for human LAMP-1 and LAMP-2 (Sawada et al., 1993) and chicken LAMP-1(Zot and Fambrough, 1990) have a conserved gene structure, each of the genes having nine exons which encode corresponding regions of the proteins.

One difference between LAMP-1 and LAMP-2 proteins is that LAMP-1 appears to be a constitutively expressed, 'housekeeping' protein (Himeno et al., 1989; Zot and Fambrough, 1990), while expression of LAMP-2 varies greatly with cell type and with developmental stage. LAMP-2, detected by antibody binding, increases following differentiation in mouse embryo carcinoma cells (Amos and Lotan, 1990). Synthesis and surface expression of LAMP-2, as defined by immunoreactivity, increases when mouse macrophages are activated (Ho and Springer, 1983). Additionally, the LAMP-2 antigen shows a limited tissue distribution (Ho and Springer, 1983). Apparently correlating with the difference in gene expression, the 5′ flanking region of the human LAMP-2 gene contains a sequence that suppresses promoter activity (Sawada et al., 1993). This suppressor sequence is lacking in both the human LAMP-1 (Sawada et al., 1993) and chicken LAMP-1 genes (Zot and Fambrough, 1990).

Although the LAMP proteins are primarily lysosomal, expression of LAMPs at the cell surface can occur as newly synthesized molecules are being transported to the lysosomes (Nabi et al., 1991; Carlsson and Fukuda, 1992; Mathews et al.,

1992) and as some molecules are cycled between lysosomes and the plasma membrane (Lippincott-Schwartz and Fambrough, 1987). Changes in the balance between lysosomal and cell surface distribution of LAMP-1 and LAMP-2 molecules as well as changes in their glycosylation patterns correlate with metastatic potential of human and murine carcinoma cells. Highly metastatic tumors show increased polylactosaminoglycans present on LAMP proteins (Laferte and Dennis, 1989; Saitoh et al., 1992) and increased surface expression of LAMP-1 and LAMP-2 (Saitoh et al., 1992). Monoclonal antibodies which bind selectively to several human carcinomas react with LAMP-1 molecules that have a modified glycosylation pattern (Hellstrom et al., 1990; Garrigues et al., 1994). Breast carcinoma cells treated with one of these antibodies show decreased cell migration (Garrigues et al. 1994). Although a pathophysiological role for the surface expression and altered glycosylation of LAMPs has been observed, the function of the LAMPs at the cell surface under normal physiological conditions is still unknown.

The initial goal of the research reported here was to determine whether a LAMP-2 homologue exists in birds and, if so, to characterize it, since cross-species comparisons often provide important insights into what stuctural features are crucial for protein function. The second goal was to determine whether, like mammalian LAMP-2, avian LAMP-2 might show an interesting pattern of cell and developmental regulation. In the course of the research we discovered that avian LAMP-2 occurs in at least three variants, LAMP-2a, b, and c. This discovery prompted exploration of additional questions: how do these variants arise? and where are the different variants expressed? The further discovery that each variant has a distinctly different cytoplasmic domain, the domain involved in targeting to lysosomes, prompted us to test the ability of each carboxyl-terminal region to function as a lysosomal targeting signal.

MATERIALS AND METHODS

cDNA cl ning

Degenerate oligonucleotide primers based on conserved amino acid sequences from mouse, rat, and human LAMP-2s, YMCNKEQ and AQDCSAD were prepared. These primers were used to amplify, by polymerase chain reaction (PCR), a 139 base pair fragment from chicken brain cDNA lambda ZAP II and Uni-ZAP libraries (Stratagene, CA). This fragment was re-amplified with ^{32}P-labeled nucleotides and used to probe plaque lifts from the same libraries. One full-length and two partial clones were obtained in this way. Overlapping cDNA clones to complete the open reading frame of the two partial cDNAs were obtained by PCR using a 5′ primer (primer K in Fig. 1) encoding sequence from the start methionine as the forward primer paired with a 3′ primer specific to each partial cDNA as the reverse primer (primers I and J in Fig. 1).

DNA sequencing

Sequencing was performed by the dideoxy chain termination method of Sanger et al. (1977) with Sequenase DNA polymerase (United States Biochemical, OH). The LAMP-2b clone and the divergent regions of the LAMP-2a and LAMP-2c clones were sequenced completely in both directions using oligonucloetide primers synthesized by a PCR-mate (Applied Biosystems, CA). The cDNA clones amplified by PCR to complete the open reading frames of the two partial clones were sequenced in one direction.

Chimera construction

The chimeras were generated by splicing the chicken LAMP-1 cDNA (LEP100) encoding the lumenal domain to the portion of the chicken LAMP-2 cDNA encoding the transmembrane domain, cytoplasmic domain and 3′ untranslated region. Restriction sites for the enzyme *HpaI* were introduced into the LEP100 and LAMP-2 cDNAs by PCR. This resulted in changing Gly363 to Val and Asp364 to Asn in LEP100 and Glu373 to Val in the LAMP-2 proteins (numbering with the start methionine as the first amino acid). The chimeras contained only the Gly363 to Val substitution. All regions of the chimeras generated by PCR were confirmed by DNA sequencing. The two

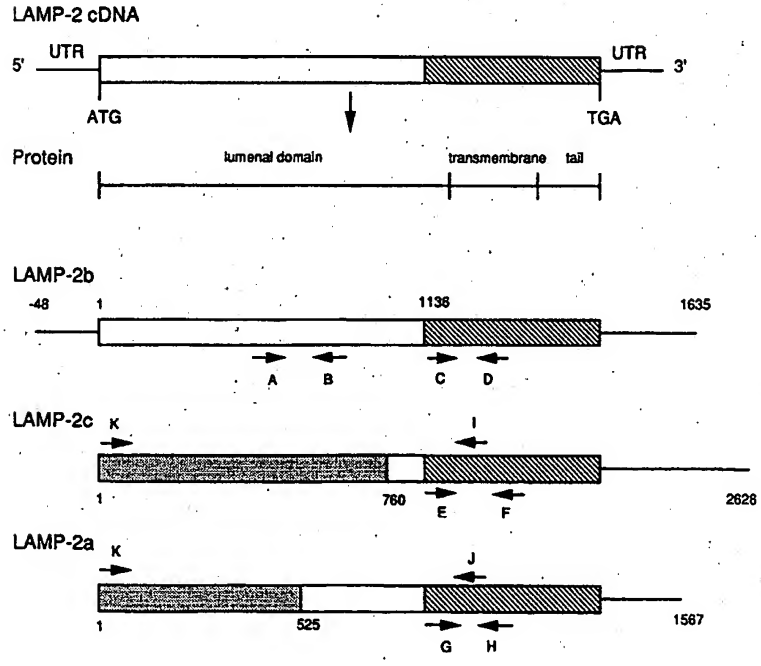

Fig. 1. Schematic diagram of the three cDNAs, including the positions of primers used for PCR. The regions unique to each cDNA are hatched. The portions of the cDNAs obtained by PCR using the K primer paired with either the I or J primer are stippled. The A and B primers were used to amplify sequences in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP to generate a probe common to the three cDNAs. The C and D, E and F, and G and H primers were used for PCR to generate ^{32}P-labeled probes specific to each cDNA.

clones were joined at the *Hpa*I site which was positioned 15 base pairs 5′ to the site of divergence among the LAMP-2s. The constructs were cloned into the pCB6 vector downstream of the human cytomegalovirus promoter for expression in mammalian cells.

Transfection and immunocytochemistry: mouse L cells were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum, gentamycin (50 μg/ml). Lipofectin (Life Technologies, MD) was used to introduce the chimeric DNA constructs into the mouse L cells, which were subsequently selected and maintained in G418 (400 μg/ml). The cells were induced with 1 mM butyrate to increase transcription directed from the human cytomegalovirus promoter for two to three days prior to processing for immunocytochemistry.

The chimeras were identified by a lumenal epitope of LEP100 recognized by the anti-LEP100 monoclonal IgG (LEP100-mAb) (Lippincott-Schwartz and Fambrough, 1986). Antibodies conjugated to fluorescein (FITC) or tetra-methyl rhodamine (TRITC) were prepared as described by Anderson and Fambrough (1983). The cells were fixed in 1% formaldehyde, permeabilized with 0.1% saponin and incubated with FITC-LEP100-mAb. Lysosomes were identified by labeling the cells with a TRITC-conjugated monoclonal antibody against mouse LAMP-1 (TRITC-mLAMP-mAb) (Chen et al., 1985). In some experiments, the cells were incubated with both FITC LEP100-mAb and TRITC-mLAMP-mAb to determine if the proteins were colocalized.

mRNA analysis: adult chicken tissues were harvested and frozen at −100°C until use. Total RNA was prepared using the RNAzol method (Cinna/ Biotecx, Texas). This is a modification of the method of RNA extraction described by Chirgwin et al. (1979). RNA (12-16 μg/lane) was electrophoresed through a 1% agarose gel containing 0.66 M formaldehyde and transferred to Genescreen Plus (NEN, MA) nylon membranes by capillary action. The RNA blots were probed with DNA fragments prepared by PCR amplification using [α-^{32}P]dATP and [α-^{32}P]dCTP. The primers used to generate the probe 5′ to the site of divergence were 5′-CCGGATCCCCCAAACCTGCTGAGAATCCAG-3′ and 5′-CCGGATCCGATCGATGTTGAGCAGAAGAG-3′ (primers A and B in Fig. 1). These primers contained sequence for *Bam*HI sites not present in the cDNA at their 5′ ends. The positions of the primers used to generate the probes specific to each divergent region are shown in Fig. 1.

RESULTS

cDNAs representing complete sequences of three forms of avian LAMP-2 were isolated from chicken brain cDNA libraries. The nucleotide sequence of the cDNA encoding LAMP-2b is shown in Fig. 2. The LAMP-2 cDNAs encode the start methionine followed by a typical signal sequence with a consensus cleavage site for signal peptidase; 92% of the deduced amino acid sequence occurs before a putative transmembrane domain, which is then followed by 11 or 12 amino acids. There are 20 consensus sequences in the lumenal domain for N-linked glycosylation (Fig. 2).

The complete open reading frames of LAMP-2a and LAMP-2c were obtained by a combination of screening plaque lifts and PCR amplification of the libraries. The nucleotide sequences of the three clones obtained from screening the chicken brain cDNA libraries are identical, with two exceptions, to base 1136 in the LAMP-2b cDNA, and then the sequences diverge. The partial cDNAs encoding LAMP-2a and -2c both have the following differences from LAMP-2b cDNA in the region conserved among the three clones: base 781 is a C instead of an A, and base 829 is a G instead of an A, changing Ile261 to Leu and Thr277 to Ala, respectively. The LAMP-2a and c cDNAs isolated by plaque lifts were obtained from a different library than LAMP-2b cDNA; thus, these codon differences in the common region are believed to reflect allelic variation rather than multiple genes. The PCR extended regions of the cDNA clones contain several random point mutations which are probably due to amplification errors. LAMP-2a contains the following point mutations: base 198 is a C instead of a T, base 421 is an A instead of a G, and base 523 is a C instead of a T. LAMP-2c contains the following mutations: base 83 is an A instead of a T and base 234 is C instead of a T. The amino acid sequences are presented in Fig. 3 with sequence identical to cLAMP-2b indicated by dashes. The nucleotide sequences of all three cDNAs are available from GenBank, accession numbers U10546, U10547, U10548.

The deduced amino acid sequences are compared with chicken LAMP-1 (LEP100), human LAMP-1, and human LAMP-2 in Fig. 3. The three cDNA clones encode proteins which fit the LAMP-2 profile, based on higher sequence identity to LAMP-2 than LAMP-1 proteins. From the start methionine to the site of divergence of the three chicken forms, the proteins are 45% identical to human LAMP-2 and only 28% identical to chicken LAMP-1. The cytoplasmic domain of chicken LAMP-2a is most similar to the reported sequences for mammalian LAMP-2 proteins with eight of twelve amino acids identical to those in human LAMP-2. Additionally, the cytoplasmic domain of each of the chicken LAMP-2 variants contains the glycine-tyrosine signal for lysosomal targeting.

The position of divergence among the three LAMP-2 clones corresponds to the position of an intron in the chicken LAMP-1, and human LAMP-1 and LAMP-2 genes (Fig. 3). Generation of the different 3′ ends of the chicken LAMP-2 cDNAs by alternative RNA splicing would be consistent with conservation of gene structure between the chicken LAMP-1 and LAMP-2 genes. Southern analysis of *Pst*I-digested genomic DNA probed with a fragment of the common region shows a single band of approximately 4 kb, suggesting that the common region is encoded by a single gene.

mRNA distribution

mRNAs from several tissues of adult chicken were analyzed on Northern blots by probing with a [^{32}P]DNA encoding a portion of the region common to LAMP-2a, b and c mRNAs (Fig. 4). The relative levels of the LAMP-2 mRNAs differ from the levels of LEP100 expressed in the same tissues. Whereas LEP100 mRNA occurs as a single 3 kb transcript in all tissues, several LAMP-2 mRNAs of different sizes were expressed in a tissue-specific manner. An mRNA which varied in abundance and migrated at ~4.4 kb was present in all of the tissues analyzed. The RNA blots were further analyzed by hybridization with probes containing regions of the cDNAs specific to each of the three clones (Fig. 5). The LAMP-2c-specific probe hybridized to the 4.4 kb mRNA that is common to all of the tissues (Fig. 5B) with the same relative intensity as detected in blots probed with a common-region probe (Fig. 4A). The LAMP-2a-specific probe hybridized to an mRNA very close to 4.4 kb; however, the mRNA was detectable only in the brain and heart (Fig. 5A). Other blots prepared from the same tissue source showed faint signals for LAMP-2a in the kidney and liver in addition to the heart and brain. The 2.5 kb

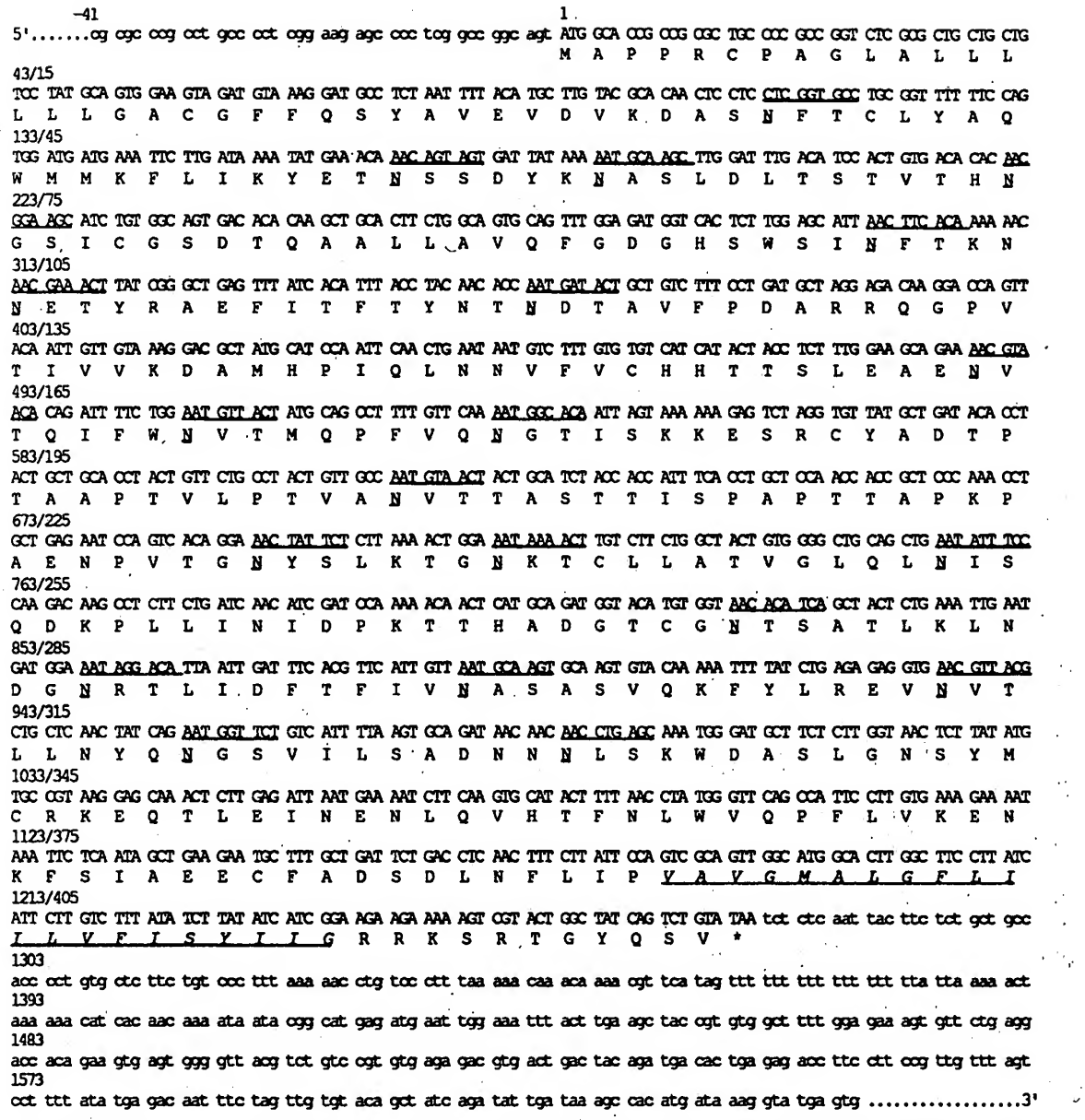

Fig. 2. The nucleotide and deduced amino acid sequence of the full-length clone LAMP-2b cDNA (GenBank accession #U10547). The two additional clones isolated by plaque lifts (GenBank accession #U10546 for LAMP-2a and #U10548 for LAMP-2c) are identical to LAMP-2b to base 1136 with two exceptions: A781 is a C which results in a Leu instead of the Ile shown, and A829 is a G resulting in an Ala instead of a Thr. After base 1136, the three clones are not conserved at either the nucleotide or amino acid level. The codons for the consensus N-linked glycosylation signals and the asparagine are underlined. The transmembrane domain is italicised and underlined.

mRNA representing a major transcript unique to adult brain was detected with the LAMP-2b-specific probe (Fig. 5C).

Functional test of variant lysosomal targeting signals

To determine whether the three variant transmembrane and cytoplasmic domains of the LAMP-2s were capable of targeting the proteins to lysosomes, chimeras were constructed. The chimeric proteins contain the lumenal domain of chicken LEP100, which is recognized by the LEP100-mAb, allowing immunocytochemical localization of the chimeric proteins expressed in mouse L cells. The transmembrane domain and the cytoplasmic domain were derived from the LAMP-2 molecules (Fig. 6). Previous studies demonstrated that the lumenal domain of LEP100 lacks targeting information. When expressed alone, the lumenal domain is secreted; fused with the transmembrane and cytosolic domains of plasma membrane proteins, it becomes localized to the plasma membrane (Mathews et al.,

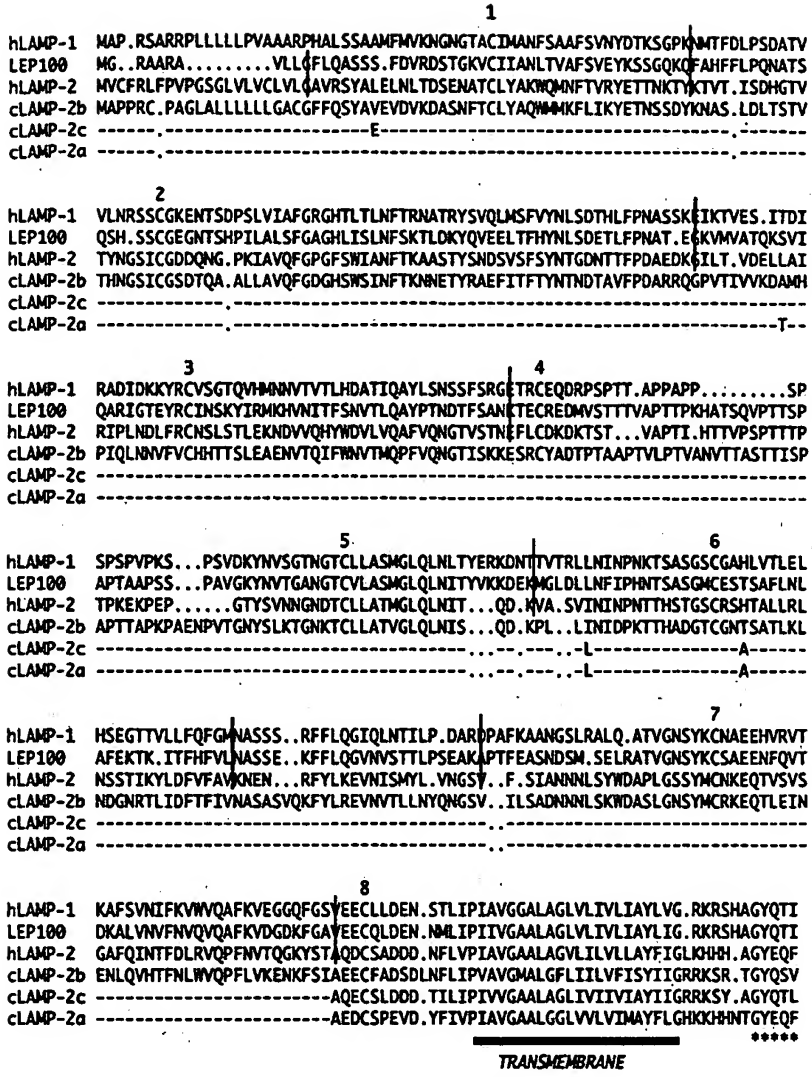

Fig. 3. A comparison of the chicken LAMP-2 amino acid sequences with chicken LEP100 and human LAMP-1 and LAMP-2. Gaps inserted to make the sequences align are indicated by dots (···). Dashes (--) indicate where the cLAMP-2 sequences are identical to LAMP-2b. The exon boundaries for chicken LEP100 (Zot and Fambrough, 1990) and human LAMP-1 and LAMP-2 (Sawada et al., 1993) are indicated by vertical lines. Where the lines pass through the amino acid, the codon is split by an intron. The conserved cysteine residues which form disulfide bridges are numbered, with cysteine residues 1 and 2, 3 and 4, 5 and 6, and 7 and 8 forming disulfide bonds. The transmembrane domains are underlined and the Gly-Tyr-X-X-hydrophobic residue motif of the lysosomal targeting signal is indicated by asterisks (*).

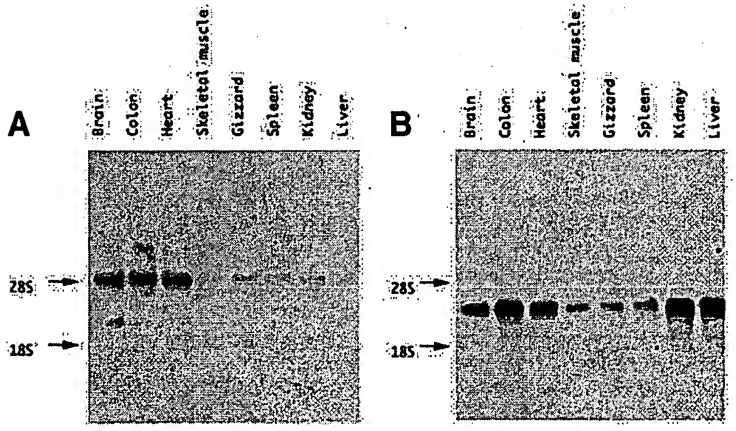

Fig. 4. Chicken total RNA analyzed for LAMP-2 (A) and LEP100 transcripts (B). A probe representing a region of the LAMP-2 cDNAs 5′ to the site of divergence was used to detect the LAMP-2 mRNAs. The probe for LEP100 represented the entire LEP100 cDNA. The same blot containing ~16 μg of total RNA per lane was probed in both A and B. The positions of the 18 and 28 S ribosomal RNA markers are indicated.

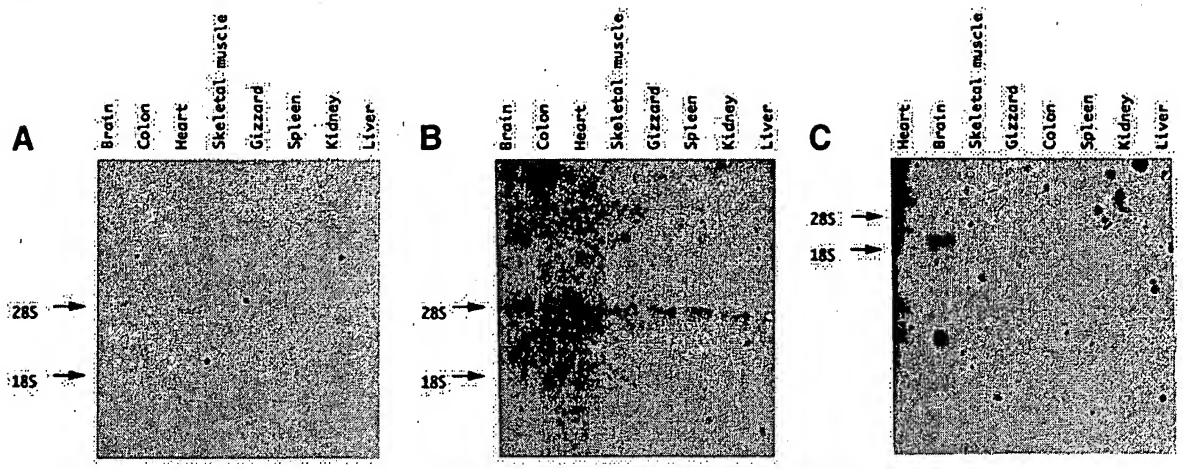

Fig. 5. The mRNAs detected by probes specific to the alternative 3′ ends of the three avian LAMP-2 cDNAs. (A) Probed with a LAMP-2a-specific sequence. (B) Probed with a LAMP-2c-specific sequence. (C) Probed with a LAMP-2b-specific sequence. The blot in A and B was the same as shown in Fig. 3. The blot in C was prepared from the same total RNA as the blot in A and B. All blots had ~16 μg of RNA per lane. The positions of the 18 and 28 S ribosomal RNA markers are indicated.

1992). Therefore, the lumenal domain does not contain information for targeting the molecule to the lysosome and, thus, any targeting information would be derived from the LAMP-2 portion of the molecule. When the transfected cells were labeled with LEP100-mAb, a pattern of fluorescent labeling typical of lysosomes was observed (Fig. 7). Double-labeling experiments show that the chimeric proteins colocalize with the endogenous mouse LAMP-1 protein, while cells not expressing the chimeric proteins do not exhibit any labeling with the LEP100-mAb; however, the lysosomes can still be visualized with the mLAMP mAb. Thus, the transmembrane and cytoplasmic domains of each of the LAMP-2 variants are sufficient to target chimeric proteins to lysosomes.

DISCUSSION

Avian LAMP-2

The deduced amino acid sequences of the new cDNA clones manifest all the hallmarks of the LAMP proteins. There is an N-terminal signal sequence probably 27 amino acids in length, followed by a 359 residue hydrophilic sequence that represents the lumenal domain. As is characteristic of LAMP proteins, this domain is rich in *N*-glycosylation sites (20) and contains 8 characteristically spaced cysteinyl residues, which form 4 disulfide bridges in the LAMP proteins (Arterburn et al., 1990). Near the middle of this domain there is a proline/threonine/serine-rich region that in the avian LAMP-2 contains 8 prolyl, 11 threonyl and 1 serinyl residue within a 38-residue span. The hydrophilic domain is followed by an extremely hydrophobic span that is the transmembrane domain in LAMP proteins. The short C-terminal hydrophilic domain begins with several basic residues and ends with the Gly-Tyr-X-X hydrophobic consensus sequence for lysosomal targeting of the LAMP proteins (Williams and Fukuda, 1990; Hunziker et al., 1991; Guarnieri et al., 1993).

The new avian LAMP appears to be the homologue of mammalian LAMP-2. The invariant region, excluding the signal sequence, shows 45% amino acid identity to human

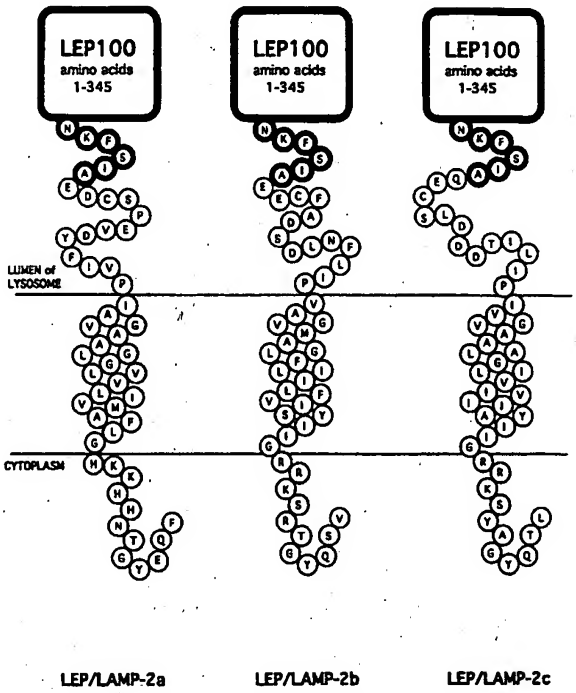

Fig. 6. A diagrammatic representation of the chimeras used for expression in mouse L cells. Amino acids 1-345 (numbering from the N-terminal phenylalanine of the mature LEP100 molecule), representing most of the lumenal portion of the chimeras, was encoded by a LEP100 cDNA. The remainder of the lumenal domain, transmembrane domain, and cytoplasmic domain was encoded by the chicken LAMP-2 cDNAs. Cysteine 8 is contributed by the lumenal domain of LAMP-2 and is assumed to be disulfide bonded to cysteine 7 of LEP100. The cDNAs were joined using a unique *Hpa*I site introduced by PCR mutagenesis.

LAMP-2 compared to 28% identity with human LAMP-1. Avian and human LAMP-1s show 46% amino acid identity in the same region. In the avian LAMP-2 variant region, amino

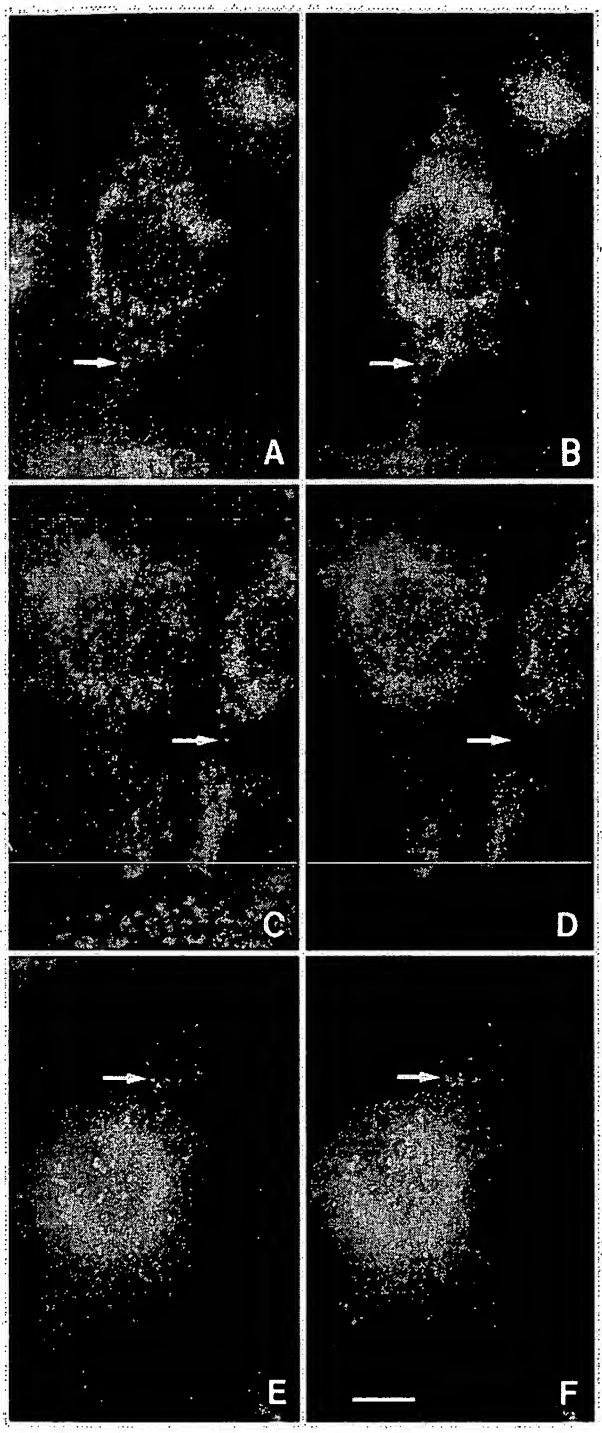

Fig. 7. Stable transfected L cells expressing LEP100/LAMP-2 chimeras double-labeled with TRITC-mLAMP-mAb and FITC-LEP100-mAb. (A and B) Cells expressing LEP/LAMP-2a. (C and D) Cells expressing LEP/LAMP-2b. (E and F) Cells expressing LEP/LAMP-2c. A, C and E were photographed with rhodamine epifluorescence. B, D and F were photographed with fluorescein epifluorescence. The arrows indicate examples of colocalization of mouse LAMP-1 and the chimera. A, C and E include portions of cells expressing endogenous mouse LAMP-1 but not the chimeric chicken protein. Bar in F, 10 μm.

acid identity between human LAMP-2 and the most similar avian homologue, LAMP-2a, is 64%. In the corresponding region of human and avian LAMP-1s, the amino acid sequence identity is 87%. The more divergent C-terminal regions of the LAMP-2s may be related to their complex pattern of expression, discussed below.

Variants of avian LAMP-2 apparently arise by alternative splicing

The nucleotide sequences of the three avian LAMP-2 cDNA clones diverge at their 3′ ends with the site of divergence located at a conserved splice junction in the human and chicken LAMP-1 and human LAMP-2 genes (Fig. 3). This suggests that the variant regions may arise by alternative splicing. Before the point of divergence, the three cDNA sequences are 98% identical. With two exceptions, the nucleotide differences lie in the sections of sequence cloned from the libraries after PCR amplification, and they may well represent errors in DNA replication during the PCR reactions. The two exceptions may represent allelic variation, since the two cDNA clones which differ from LAMP-2b are identical to each other and were obtained from a different library than the LAMP-2b clone. Notably, only two differences at codon third-base positions were observed, both from PCR-generated DNA. Since third-base sequences are frequently free from selective pressures because of degeneracy in the genetic code, the maintenance of these nucleotides in the three avian LAMP-2 cDNAs strongly suggests that the common region is encoded by a single gene. Analysis of digested genomic DNA also suggests that there is a single gene. Therefore, we propose that the variant regions are encoded by alternative exons at the 3′ end of the avian LAMP-2 gene.

Avian LAMP-2s are expressed in a tissue-specific manner

Analysis of the mRNAs from various chicken tissues demonstrated that the heart and brain contain differentially expressed mRNAs. A probe to the invariant region of avian LAMP-2 mRNA hybridized with five mRNAs in adult brain, one of which was unique to the brain. Four of the five mRNAs detected in the brain were also expressed in adult heart. The two largest mRNAs present in heart and brain were not represented by any of the cDNAs described and represent minor mRNAs. It is not known whether these represent additonal splice variants of the LAMP-2 transcript. Every tissue analyzed expressed an mRNA that was detected with a probe specific for the LAMP-2c cDNA. A LAMP-2b-specific probe hybridized with an abundant LAMP-2 mRNA species found only in the brain. These results are consistent with the notion that, unlike LAMP-1, LAMP-2 is regulated in a tissue-specific manner (Sawada et al., 1993).

The C-terminal end of avian LAMP-2 contains lys s mal targ ting inf rmati n

The three avian LAMP-2s differ in the region of the molecule that, in other LAMPs, contains the lysosomal targeting signal.

Given the complete amino acid sequence identity between human and avian LAMP-1 in the targeting signals, we were surprised to find three such sequences in avian LAMP-2, none of which is exactly like the human homologue. When the three variant regions were expressed in mouse L cells as chimeras with the ectodomain of avian LAMP-1 (LEP100), all the chimeras were found to localize to a great extent in lysosomes. These results strongly support our conclusion that the new cDNAs encode LAMP proteins. However, why should there be three alternative targeting signals for avian LAMP-2? One possible explanation is that these alternative C-terminal ends might be involved more subtly in differential intracellular targeting or subcellular distribution. Such a role might help to explain the altered distributions of LAMP molecules that occur in some situations, including macrophage activation (Ho and Springer, 1983) and increased metastatic potential of tumor cells (Laferte and Dennis, 1989; Hellstrom et al., 1990; Saitoh et al., 1992; Garrigues et al., 1994). Our results with the chimeras are not sufficiently quantitative to serve as a test of this hypothesis. Furthermore, in the tissues where they are expressed endogenously, the LAMP-2 variants may show different cellular distributions. Testing this idea awaits the development of variant-specific antibodies.

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REFERENCES

Amos, B. and Lotan, R. (1990). Modulation of lysosomal-associated membrane glycoproteins during retinoic acid-induced embryonal carcinoma cell differentiation. *J. Biol. Chem.* **265**, 19192-19198.

Anderson, M. J. and Fambrough, D. M. (1983). Aggregates of acetylcholine receptors are associated with plaques of a basal lamina heparin sulfate proteoglycan on the surface of skeletal muscle fibers. *J. Cell Biol.* **97**, 1396-1411.

Arterburn, L. M., Earles, B. J. and August, J. T. (1990). The disulfide structure of mouse lysosome-associated membrane protein 1. *J. Biol. Chem.* **265**, 7419-7423.

Carlsson, S. R. and Fukuda, M. (1992). The lysosomal membrane glycoprotein lamp-1 is transported to lysosomes by two alternative pathways. *Arch. Biochem. Biophys.* **296**, 630-639.

Cha, Y., Holland, S. M. and August, J. T. (1990). The cDNA sequence of mouse LAMP-2. *J. Biol. Chem.* **265**, 5008-5013.

Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I. and August, J. T. (1985). Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* **101**, 85-95.

Chen, J. W., Cha, Y., Yuksel, K. U., Gracy, R. W. and August, J. T. (1988). Isolation and sequencing of a cDNA clone encoding lysosomal membrane glycoprotein mouse LAMP-1. *J. Biol. Chem.* **263**, 8754-8758.

Chirgwin, J. M., Przybyla, R. J., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5300.

Fambrough, D. M., Takeyasu, K., Lippincott-Schwartz, J. and Siegel, N. R. (1988). Structure of LEP100, a glycoprotein that shuttles between lysosomes and the plasma membrane, deduced from the nucleotide sequence of the encoding cDNA. *J. Cell Biol.* **106**, 61-67.

Fukuda, M., Viitala, J., Matteson, J. and Carlsson, S. R. (1988). Cloning of cDNAs encoding human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. *J. Biol. Chem.* **263**, 18920-18928.

Fukuda, M. (1991). Lysosomal membrane glycoproteins. *J. Biol. Chem.* **266**, 21327-21330.

Garrigues, J., Anderson, J., Hellstrom, K. E. and Hellstrom, I. (1994). Anti-tumor antibody BR96 blocks cell migration and binds to a lysosomal membrane glycoprotein on cell surface microspikes and ruffled membranes. *J. Cell Biol.* **125**, 129-142.

Granger, B. L., Green, S. A., Gabel, C. A., Howe, C. L., Mellman, I. and Helenius, A. (1990). Characterization and cloning of lgp110, a lysosomal membrane glycoprotein from mouse and rat cells. *J. Biol. Chem.* **265**, 12036-12043.

Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y. and August, J. T. (1993). The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein-1. *J. Biol. Chem.* **268**, 1941-1946.

Hellstrom, I., Garrigues, H. J., Garrigues, U. and Hellstrom, K. E. (1990). Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le^y-related cell surface antigens. *Cancer Res.* **50**, 2183-2190.

Himeno, M., Noguchi, Y., Sasaki, H., Tanaka, Y., Furano, K., Kono, A., Sakaki, Y. and Kato, K. (1989). Isolation and sequencing of a cDNA clone encoding 107 kDa sialoglycoprotein in rat liver lysosomal membranes. *FEBS Lett.* **244**, 351-356.

Ho, M.-K. and Springer, T. A. (1983). Tissue distribution, structural characterization and biosynthesis of Mac-3, a macrophage surface glycoprotein exhibiting molecular weight heterogeneity. *J. Biol. Chem.* **258**, 636-642.

Howe, C. L., Granger, B. L., Hull, M., Green, S. A., Gabel, C. A., Helenius, A. and Mellman, I. (1988). Derived protein sequence, oligosaccharides, and membrane insertion of the 120-kDa lysosomal membrane glycoprotein (lgp120): identification of a highly conserved family of lysosomal membrane glycoproteins. *Proc. Nat. Acad. Sci. USA* **85**, 7577-7581.

Hunziker, W., Harter, C., Matter, K. and Mellman, I. (1991). Basolateral sorting in MDCK cells requires a distinct cytoplasmic domain determinant. *Cell* **66**, 907-920.

Kornfeld, S. and Mellman, I. (1989). The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**, 483-525.

Laferte, S. and Dennis, J. W. (1989). Purification of two glycoproteins expressing β1-6 branched Asn-linked oligosaccharides from metastatic tumour cells. *Biochem. J.* **259**, 569-576.

Lippincott-Schwartz, J. and Fambrough, D. M. (1986). Lysosomal membrane dynamics: structure and interorganellar movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* **102**, 1593-1605.

Lippincott-Schwartz, J. and Fambrough, D. M. (1987). Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. *Cell* **49**, 669-677.

Mathews, P. M., Martinie, J. B. and Fambrough, D. M. (1992). The pathway and targeting signal for delivery of the integral membrane glycoprotein LEP100 to lysosomes. *J. Cell Biol.* **118**, 1027-1040.

Nabi, I. R., Le Bivic, A., Fambrough, D. and Rodriguez-Boulan, E. (1991). An endogenous MDCK lysosomal membrane glycoprotein is targeted basolaterally before delivery to lysosomes. *J. Cell Biol.* **115**, 1573-1584.

Noguchi, Y., Himeno, M., Sasaki, H., Tanaka, Y., Kono, A., Sakaki, Y. and Kato, K. (1989). Isolation and sequencing of a cDNA clone encoding 96 kDa sialoglycoprotein in rat liver lysosomal membranes. *Biochem. Biophys. Res. Commun.* **164**, 1113-1120.

Saitoh, O., Wang, W.-C., Lotan, R. and Fukuda, M. (1992). Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. *J. Biol. Chem.* **267**, 5700-5711.

Sanger, F., Nicklen, S. and Coulsen, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.

Sawada, R., Jardine, K. A. and Fukuda, M. (1993). The genes of major lysosomal membrane glycoproteins, Lamp-1 and Lamp-2. *J. Biol. Chem.* **268**, 9014-9022.

Viitala, J., Carlsson, S. R., Siebert, P. D. and Fukuda, M. (1988). Molecular cloning of cDNAs encoding lamp A, a human lysosomal membrane glycoprotein with apparent Mr=120,000. *Proc. Nat. Acad. Sci. USA* **85**, 3743-3747.

Williams, M. A. and Fukuda, M. (1990). Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol.* **111**, 955-966.

Zot, A. S. and Fambrough, D. M. (1990). Structure of a gene for a lysosomal membrane glycoprotein (LEP100). *J. Biol. Chem.* **265**, 20988-20995.

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Intracellular Sorting and Targeting of Melanosomal Membrane Proteins: Identification of Signals for Sorting of the Human Brown Locus Protein, GP75

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Abstract. The structural and functional integrity of cytoplasmic organelles is maintained by intracellular mechanisms that sort and target newly synthesized proteins to their appropriate cellular locations. In melanocytic cells, melanin pigment is synthesized in specialized organelles, melanosomes. A family of melanocyte-specific proteins, known as tyrosinase-related proteins that regulate melanin pigment synthesis, is localized to the melanosomal membrane. The human brown locus protein, tyrosinase-related protein-1 or gp75, is the most abundant glycoprotein in melanocytic cells, and is a prototype for melanosomal membrane proteins. To investigate the signals that allow intracellular retention and sorting of glycoprotein (gp)75, we constructed protein chimeras containing the amino-terminal extracellular domain of the T lymphocyte surface protein CD8, and transmembrane and cytoplasmic domains of gp75. In fibroblast transfectants, chimeric CD8 molecules containing the 36–amino acid cytoplasmic domain of gp75 were retained in cytoplasmic organelles. Signals in the gp75 cytoplasmic tail alone, were sufficient for intracellular retention and targeting of the chimeric proteins to the endosomal/lysosomal compartment. Analysis of subcellular localization of carboxyl-terminal deletion mutants of gp75 and the CD8/gp75 chimeras showed that deletion of up to 20 amino acids from the gp75 carboxyl terminus did not affect intracellular retention and sorting, whereas both gp75 and CD8/gp75 mutants lacking the carboxyl-terminal 27 amino acids were transported to the cell surface. This region contains the amino acid sequence, *asn-gln-pro-leu-leu-thr*, and this hexapeptide is conserved among other melanosomal proteins. Further evidence showed that this hexapeptide sequence is necessary for intracellular sorting of gp75 in melanocytic cells, and suggested that a signal for sorting melanosomal proteins along the endosomal/lysosomal pathway lies within this sequence. These data provide evidence for common signals for intracellular sorting of melanosomal and lysosomal proteins, and support the notion that lysosomes and melanosomes share a common endosomal pathway of biogenesis.

BIOGENESIS of cytoplasmic organelles requires targeting of soluble and membrane proteins to appropriate vesicular precursors. Signals responsible for intracellular sorting and targeting of newly synthesized proteins to various organelles have been identified, including those for lysosomes and peroxisomes. Melanosomes, the site of melanin pigment synthesis, are specialized organelles present in melanocytic cells that produce pigment. Biogenesis of melanosomes is not well understood (Novikoff et al., 1968; Maul and Brumbaugh, 1971). Based on electron microscopic histochemical studies, it has been proposed that tyrosinase, the critical and rate-limiting enzyme for melanin synthesis, is transported by coated vesicles from Golgi to premelanosomal vesicles (Novikoff et al., 1968; Chakraborty et al., 1989; Moellmann et al., 1989; Zhou et al., 1993). However, signals responsible for sorting tyrosinase and other melanosomal membrane proteins for intracellular retention and targeting to premelanosomal vesicles have not been investigated.

Recently, several proteins that influence melanin pigmentation have been identified. These pigmentation-associated proteins include a family of proteins known as tyrosinase-related proteins (TRPs)[1] that are encoded by genes that map to different genetic loci that determine coat color of mice (Shibahara et al., 1986; Jackson, 1988; Vijayasaradhi et al., 1990; Kwon et al., 1991; Gardner et al., 1992; Jackson et al., 1992; Tsukamoto et al., 1992; Rinchik et al., 1993). The product of the *brown* (*b*) locus gene, is a 75-kD

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1. *Abbreviations used in this paper*: Endo H, endoglycosidase H; gp, glycoprotein; lgps, lysosomal glycoproteins; TM, transmembrane; TRP, tyrosinase-related protein.

glycoprotein known as TRP-1 or glycoprotein (gp)75. The *b* locus protein catalyzes the oxidation of 5,6-dihydroxyindole-2-carboxylic acid, an intermediate in the melanin synthesis pathway, and mutations and allelic differences at the *b* locus produce shades of brown coat color in mice (Silvers, 1979; Zdarsky et al., 1990; Jiménez-Cervantes et al., 1994). The *b* locus is of particular interest because it is known to influence the structure of melanosomes (Moyer, 1963, 1966; Foster, 1965; Rittenhouse, 1968; Hearing et al., 1973).

The *b* locus product, gp75, is highly conserved between mouse and human, and shares a 43% amino acid sequence identity with tyrosinase. In human melanocytic cells, gp75 is the most abundant glycoprotein (Vijayasaradhi et al., 1991; Tai et al., 1983). The biosynthesis and intracellular movement of gp75 has been studied in detail. gp75 is synthesized as a 55-kD polypeptide, glycosylated by addition and processing of at least five Asn-linked oligosaccharides through the *cis*- and *trans*-Golgi complex, and transported to melanosomes as a mature 75-kD protein (Vijayasaradhi et al., 1991). To identify the signals for intracellular sorting and targeting of gp75, we constructed chimeric proteins consisting of the extracellular domain of the T lymphocyte surface glycoprotein CD8, transmembrane (TM) domains of CD8 or gp75, and the cytoplasmic tail (Cyt) of gp75, and studied their cellular localization. Our data showed that the presence of the gp75 cytoplasmic tail was sufficient to cause intracellular retention of the chimeric CD8 molecules. Analysis of carboxyl-terminal deletion mutants of gp75 and chimeric CD8/gp75 showed that a sequence of six amino acids within the cytoplasmic tail of gp75 was necessary for sorting gp75 along the endocytic pathway in fibroblast transfectants. This peptide sequence is conserved between mouse and human gp75 and tyrosinase, and residues within this sequence are also conserved among other melanocyte-specific proteins known to be involved in regulation of melanin pigmentation.

Materials and Methods

Cells and Antibodies

Human melanoma cells SK-MEL-19, SK-MEL-23 clone 22a, and mouse L cell fibroblasts were cultured as described earlier (Bouchard et al., 1989; Vijayasaradhi et al., 1991). Mouse hybridoma secreting anti-human CD8 mAb OKT-8 was obtained from American Type Culture Collection (Rockville, MD).

Purified mouse anti-human gp75 mAb TA99 IgG used for this study was described earlier (Vijayasaradhi et al., 1990). Undiluted tissue culture supernatant of hybridoma OKT-8 collected after 48–72 h was used for staining CD8. Anti-mouse lysosomal membrane glycoprotein LAMP-1 antibody 1D4B (Chen et al., 1985) was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA).

Cloning and Expression of Full-length gp75 cDNA

A full-length 2.8-kb EcoRI fragment was isolated from a human melanoma cDNA library, and subcloned into the unique EcoRI site of eukaryotic expression vectors pCEXV3 (Bouchard et al., 1989) or pSVK3.1 (a derivative of vector pSVK3 obtained by deletion of the SacI fragment within the multiple cloning site), or SmaI site of pSVK3 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) after a fill-in reaction with Klenow fragment of DNA polymerase (New England Biolabs, Beverly, MA). The orientation of the cloned insert was determined by restriction analysis, and confirmed by dideoxy chain termination sequencing method (Sequenase kit, United States Biochemical Corp., Cleveland, OH) using an oligonucleotide primer complementary to the vector sequences upstream of the cloning site.

Mouse L cell fibroblasts were transfected with plasmid containing gp75 cDNA and pSV2neo. Transfected clones were isolated by selecting for growth in the antibiotic G418 (1 mg/ml; GIBCO BRL, Gaithersburg, MD), and screened for gp75 expression by immunofluorescence staining with the mAb TA99 (Vijayasaradhi et al., 1991).

Construction of CD8/gp75 Chimeric cDNA Expression Plasmids

The plasmid EBO-pCD-Leu2 containing human CD8-α cDNA was obtained from American Type Culture Collection (Margolskee et al., 1988). The 2.3-kb BamHI fragment from this plasmid was isolated, made blunt-ended with Klenow fragment, and cloned into the SmaI site of the expression vector pSVK3. The orientation of the cDNA insert in the recombinant plasmids in *Escherichia coli* DH5-α was analyzed by appropriate restriction enzyme digestions, and confirmed by DNA sequencing.

Chimeric cDNAs encoding fusion proteins CD8/gp75(TM+Cyt), and CD8/gp75(Cyt) were constructed by the following methods. First, appropriate restriction sites at or near the TM/Cyt junction of CD8, and luminal/TM and TM/Cyt junctions of gp75 were generated by site-directed mutagenesis (Kunkel et al., 1987) using Mutagene kit (Bio-Rad Laboratories, Hercules, CA). Specifically, a mutant gp75 plasmid pSVgp75RV was generated by introducing an EcoRV restriction site at nucleotide 1560 (luminal/TM junction) of gp75 cDNA in plasmid pSVK3 using the mutagenic oligonucleotide 5′-TACTGCTATGGCAATGATATCAGGTACACTA-3 (mutations introduced are shown in bold). This resulted in the conversion of glutamic acid at position 477 (amino acids numbered starting with the methionine coded by the initiation codon) to aspartic acid. Mutant plasmids pSVgp75H and pSVleu2H were generated by introducing a HindIII restriction site in gp75 cDNA at nucleotide 1627, (gp75 TM/Cyt junction), and at nucleotide 706 (CD8 TM/Cyt junction) in CD8 cDNA using the mutagenic oligonucleotides 5′-GCGTCTGGCACGAAGCTTATAAGAAGCAGT-3′ and 5′-GTCTTCGGTTCCTAAGCTTGCAGTAAAGGGT-3′, respectively. This resulted in conversion of leucine at position 500 to lysine, and isoleucine at 501 to leucine in gp75; and asparagine at 207 to lysine, and histidine at 208 to proline in CD8. Mutants were first identified by appropriate restriction enzyme digestion and confirmed by sequencing the relevant regions of the plasmids using Sequenase sequencing kit. Transient expression in mouse fibroblasts and immunofluorescence analyses with mAbs TA99 (anti-gp75) and OKT-8 (anti-human CD8) showed that intracellular staining of mutant proteins was identical to the distribution of wild-type counterparts, i.e., punctate cytoplasmic staining of gp75 and cell-surface expression of CD8.

Plasmid pSVgp75RV was digested with EcoRV and XbaI to produce an ~1.2-kb fragment containing the TM+Cyt sequence and 3′ untranslated sequence of gp75 cDNA, including part of the multiple cloning site sequences of the vector; plasmid pSVleu2H was digested with EcoRV and XbaI and the large ~4-kb plasmid DNA fragment lacking TM and Cyt sequences of CD8 cDNA was isolated. The 1.2-kb EcoRV-XbaI gp75 fragment was ligated with the large EcoRV-XbaI pSVleu2H fragment to generate a plasmid construct encoding the fusion protein CD8/gp75(TM+Cyt). Similarly, an ~1-kb HindIII–XbaI gp75 cDNA fragment (containing gp75 Cyt and 3′ untranslated sequences), and an ~4-kb HindIII–XbaI CD8 cDNA plasmid fragment (lacking the cytoplasmic tail sequences of CD8) were isolated, respectively, from plasmids pSVgp75H and pSVleu2H, and ligated to generate the fusion protein CD8/gp75(Cyt). Regions of the plasmids at the CD8/gp75 junctions were sequenced from at least two independent clones to confirm the restoration of the reading frame. Large-scale plasmid preparations (QIAGEN, Inc., Chatsworth, CA) were further verified by restriction enzyme digestions for the presence of enzyme sites unique to gp75 and CD8 at appropriate regions in the chimeric plasmids.

Generation of gp75 Carboxyl-terminal Deletion Mutants

pSVK3.1gp75 was used to generate carboxyl-terminal deletion mutants. The restriction enzyme site BglII at nucleotide 2000 of gp75 cDNA is a unique site within the plasmid pSVK3.1. Plasmid pSVK3.1gp75 (10 μg) was linearized by digestion with 40 U of BglII in a 50-μl reaction for 3 h at 37°C. Linearized ~6.7-kb DNA was then digested for 3–4 min with Bal 31 nuclease (1 U enzyme/μg DNA) in a 50-μl reaction. Digested DNA was immediately extracted with phenol/chloroform to inactivate and remove the nuclease, and the ends were filled in by Klenow fragment of DNA

polymerase I to increase the population of blunt-ended molecules (Sambrook et al., 1989). Klenow fragment was inactivated by heating at 75°C for 10 min, and a suppressible reading frame termination linker containing restriction site NheI, 5′-CTAGCTAGCTAG-3′ (Pharmacia LKB Biotechnology Inc.), was ligated to the blunt-ended, truncated pSVK3.1gp75 DNA molecules with 1 U of T4 DNA ligase in a 20-μl reaction for 3 h at room temperature. The ligation mixture was used to transform *E. coli* strain DH5-α. Ampicillin-resistant bacterial colonies were analyzed by agarose gel slot lysis method for the presence of plasmid DNA of appropriate size. Plasmid DNA from 15 transformants was isolated, analyzed by restriction enzyme digestion, and partially sequenced to determine the number of bases deleted from the carboxyl terminus, and to confirm the addition of termination linker.

Transfection and Transient Expression

A transient transfection method was developed and optimized to study the intracellular distribution of gp75 expressed by mutant constructs. Briefly, 2–4 × 10^4 SK-MEL-23 clone 22a melanoma cells and mouse L cells fibroblasts were plated in 8-well chamber slides (Lab-Tek, Naperville, IL). The cells were transfected with plasmid DNA by calcium phosphate precipitate method for 16–24 h, and then allowed to accumulate the expressed protein for 12–48 h.

Immunofluorescence Microscopy

Cells on the 8-well glass slides were fixed with formaldehyde, followed by methanol, and stained with gp75-specific mouse mAb TA99 or OKT-8 followed by FITC-conjugated anti-mouse IgG. Cells were examined under fluorescence microscope (Optiphot; Nikon Inc., Instrument Group, Melville, NY) and photographed using Ektachrome film (Eastman Kodak Co., Rochester, NY).

Immunoelectronmicroscopy

mAb TA99 directly conjugated to 10-nm gold particles was used for localization of gp75 by immunoelectron microscopy. Colloidal gold was prepared as described (Smit and Todd, 1986) and mAb TA99–gold conjugate was prepared according to Alexander et al. (1985).

Human melanoma SK-MEL-19 cells were fixed with 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, infused with 2.3 M sucrose in PBS, and the cell pellet was then frozen in liquid nitrogen. Ultrathin sections were cut and collected on Formvar-carbon coated nickel grids. The sections on the grids were incubated in 0.5% BSA in PBS to block nonspecific protein binding sites, and then stained with mAb TA99 conjugated to 10 nm gold particles. Washing and staining of the sections were performed according to Griffiths et al. (1983). Sections were observed on an electron microscope (100CX; JEOL USA Analytical Instruments, Cranford, NJ).

Metabolic Pulse–Chase Labeling, Endoglycosidase H (Endo H) Digestion, Immunoprecipitation, and SDS-PAGE Analysis

Transfected fibroblasts grown in 25-cm^2 tissue culture flasks were labeled for 30 min, and for pulse–chase analysis, SK-MEL-19 melanoma cells and transfected fibroblasts were labeled 5 min with 100 μCi of [^{35}S] methionine (EXPRE^{35}S^{35}S protein labeling mix; New England Nuclear, Boston, MA), washed twice, and chased in normal growth medium containing cold methionine for indicated periods. Cells were harvested, washed twice with ice-cold PBS, and lysed in 150–200 μl lysis buffer (10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin, 0.5 mg/ml leupeptin, and 0.2 mM PMSF). The lysates were cleared by centrifugation at 15,000 *g* for 10 min at 4°C, and stored at −20°C. Incorporation of radioactivity into proteins was determined by precipitation with TCA. Briefly, 1–2 μl of cell lysate was spotted on glass fiber filters (Whatman Inc., Clifton, NJ) on multifiltration apparatus (Millipore Corp., Bedford, MA), and each filter was washed with 4 × 4 ml ice-cold 10% TCA, dehydrated with absolute ethanol, and air-dried. Protein-bound radioactivity was measured in Liquiscint (National Diagnostics Inc., Manville, NJ) in a scintillation counter.

Appropriate volumes of cell lysates containing 1–2 × 10^6 TCA-insoluble cpm were made up to 0.2 ml with lysis buffer, and incubated with mAb TA99 (300 μg/ml diluted in gamma globulin free fetal bovine serum) for 1–2 h at 4°C. At the end of incubation with the primary antibody, a 40–50-μl suspension of rabbit anti-mouse IgG coupled to protein A Sepharose (10% wt/vol) was added and incubated for 2–4 h or overnight at 4°C. Immunoprecipitates were washed eight times with 10 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and 1% NP-40 (TNEN), four times with one-tenth strength TNEN containing 0.5 M NaCl, and finally with distilled water (Vijayasaradhi et al., 1991).

For Endo H digestion, the immunoprecipitates were dissociated by suspending in 20 μl 0.2% SDS, heated for 5 min at 100°C, and diluted with 20 μl 0.1 M sodium citrate buffer, pH 5.5, Endo H, 50 mU/ml; digestion was carried out at 37°C for 18–24 h. The reaction mixture was layered over with 50 μl toluene. Control tubes were treated similarly, except that equal volume of 0.05 M sodium citrate buffer, pH 5.5, was added instead of Endo H.

Proteins were analyzed by 9% SDS-PAGE. Radioactive protein bands were visualized by fluorography. Prestained protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) are lysozyme, 15,000; β-lactoglobulin, 18,000; α-chymotrypsinogen, 25,000; ovalbumin, 42,000; BSA, 66,000; phosphorylase B, 104,000; myosin (H-chain), 199,000.

Results

Human gp75 cDNA encodes a 537–amino acid-long polypeptide consisting of amino-terminal signal peptide sequence, a long amino-terminal domain, a TM region, and a 36–amino acid-long carboxyl-terminal domain (Fig. 1). Light and electron microscopic localization studies showed that in melanocytic cells, gp75 is localized to juxtanuclear membranes and melanosomes (Fig. 2). Very little, or no expression of gp75 could be detected on the cell surface of human melanoma cells using sensitive techniques, such as mixed hemadsorption assay and cell-surface labeling with radioactive iodine (data not shown). To investigate the protein structural requirements for intracellular sorting and tar-

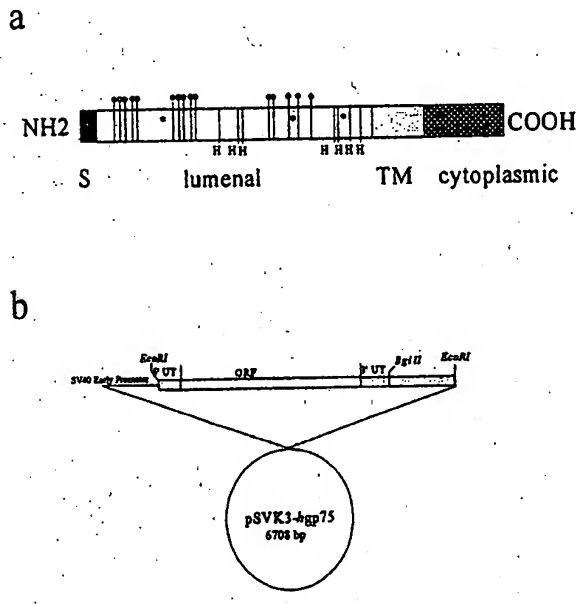

Figure 1. Schematic diagrams of (*a*) gp75 showing the protein domains and the structural features conserved among members of the tyrosinase-related family, and (*b*) expression vector containing gp75 cDNA with the restriction sites relevant to this study. (*a*) S, signal peptide; TM, transmembrane region. Vertical bars projecting up with a dot, conserved cysteine residues. Vertical bars projecting down, conserved histidines (required for binding copper). Asterisks, conserved Asn-linked glycosylation acceptor sites. (*b*) ORF, open reading frame; UT, untranslated region. Bars and circle not drawn to scale.

Figure 2. Subcellular distribution of gp75. Immunoelectron microscopic analysis of human melanoma cells SK-MEL-19 using 10-nm gold particles conjugated to mAb TA99. (*Arrows*) gp75 localized to the melanosomal membrane. (*Left inset*) A high magnification view of the melanosome marked by arrow shows the contiguous melanosomal membrane (*arrowheads*) to which gp75 is localized. (*Right inset*) Immunofluorescent staining of SK-MEL-19 cell with mAb TA99 showing bright juxtanuclear staining and the distribution of gp75 in cytoplasmic vesicles. Bars, 0.25 μm.

geting of gp75 to melanosomes, we constructed protein chimeras, generated deletion mutants, and studied the subcellular distribution of mutant and chimeric proteins transiently expressed in fibroblasts.

Because we hypothesized that the carboxyl tail of gp75 contained a sorting signal, we first had to investigate the membrane orientation of gp75. Postnuclear membranes from metabolically labeled melanoma cells were digested with proteinase K or trypsin before detergent solubilization and immunoprecipitation with mAb TA99. SDS-PAGE analysis showed a broad band of 65–70 kD, ~5 kD smaller than the undigested gp75 protein (data not shown). This is consistent with a membrane orientation of gp75 in which the bulk of the protein, with its glycosylated amino-terminal domain, is inside the melanosomal lumen (inaccessible to the proteases) and the ~5-kD short carboxyl tail is located outside, facing the cytosol.

Next, we established that biosynthesis and processing of gp75 is similar in fibroblasts and melanocytic cells. Mouse L cell fibroblasts were cotransfected with gp75 cDNA and pSV2neo plasmid, and G418-resistant clones that stained positive with mAb TA99 were isolated. Immunoprecipitation analysis of the cell lysates of transfectants metabolically labeled with [^{35}S]methionine showed that mAb TA99 precipitated a broad 72–75-kD band identical to melanoma gp75. mAb TA99 recognizes conformational epitopes on glycosylated gp75 (Vijayasaradhi et al., 1991), so the ability of mAb TA99 to precipitate gp75 from the transfectants demonstrated that the protein synthesized in mouse fibroblasts was structurally similar to the human melanoma gp75.

We compared the time-course of intracellular movement of gp75 in transfected fibroblasts and human melanoma cells. Fig. 3 shows pulse–chase analyses of posttranslational processing of gp75 in SK-MEL-19 melanoma cells and transfected fibroblast clone. At the end of 5 min pulse labeling, newly synthesized gp75 appeared as a major 69-kD band (and a minor 67-kD band) in melanoma cells, and a single 69-kD band in fibroblasts. Digestion with Endo H, which removes high mannose Asn-linked oligosaccharides, produced a 55-kD core polypeptide band showing that the 69- and 67-kD bands represented the ER and *cis*-Golgi forms of gp75 with high mannose oligosaccharide chains. With increasing periods of chase, the 55-kD band produced by Endo H digestion became less prominent with concomitant appearance of bands having reduced electrophoretic mobility. After 2 h chase, in both melanoma cells and transfected fibroblasts, Endo H digestion produced only the higher mol wt band(s) but not the 55-kD band. These data showed that processing through the medial- and *trans*-Golgi and maturation of gp75 in fibroblasts occurred at a rate similar to that in melanocytic cells.

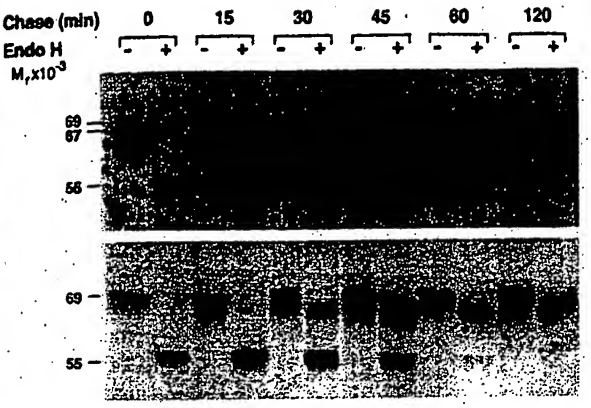

Figure 3. Pulse-chase analysis of oligosaccharide processing of gp75 in human melanoma cells SK-MEL-19 (*upper panel*) and clone 7-transfected fibroblasts (*lower panel*). Cells were pulse labeled with [^{35}S]methionine for 5 min and chased for increasing lengths of indicated time. gp75 protein was immunoprecipitated and analyzed by SDS-PAGE with (+) or without (−) Endo H digestion. Numbers on the left are apparent molecular masses (*kD*) of the protein bands.

Colocalization of gp75 and Lysosomal Membrane Proteins in Transfected Fibroblasts

Indirect immunofluorescence staining of transfected fibroblasts with mAb TA99 showed intracellular juxtanuclear or perinuclear staining, and a punctate cytoplasmic staining similar to the pattern observed in human melanocytic cells. In melanocytic cells, the punctate cytoplasmic staining represented gp75 targeted to melanosomes and their precursors. Double immunofluorescence staining of fibroblast transfectants with mAb TA99 and anti–LAMP-1 antibody 1D4B showed that almost all intracellular structures that stained positive for melanosomal gp75 also stained with the antibody to LAMP-1 (Fig. 4). The targeting of gp75 to LAMP-1–positive structures showed that, in fibroblasts, gp75 was sorted along the endosomal/lysosomal pathways. This is consistent with the observation that in 3T3-fibroblast transfectants, mouse gp75 was localized to the LAMP-1– and β-glucuronidase–positive endosomal/lysosomal structures (Winder et al., 1993). These data showed that signals responsible for sorting and targeting of gp75 to intracellular vesicles (melanosomes in melanocytic cells) are also functional in fibroblasts.

Cytoplasmic Domain of gp75 Influences the Cellular Localization of CD8/gp75 Chimeras

To localize the signals responsible for intracellular sorting and targeting of gp75 to lysosomes, we constructed chimeric proteins consisting of the extracellular domain of the T lymphocyte surface protein CD8 and the TM and cytoplasmic domains of gp75 (CD8/gp75 TM+Cyt), and the extracellular and TM domains of CD8 and the cytoplasmic domain of gp75 (CD8/gp75 cyt) (Fig. 5). Cellular localization of wild-type CD8, CD8 protein lacking the cytoplasmic tail (tailless CD8), and the CD8/gp75 chimeric proteins expressed in fibroblasts was studied by immunofluorescence staining with mAb OKT-8, which recognizes epitope(s) on the extracellular domain of CD8 molecule.

Membrane-permeabilized fibroblasts expressing full-length wild-type CD8 showed a pattern of staining that demarcated the cellular margins, with a diffuse staining over the cell body and occasional patches of intense staining near the margins (Fig. 6 *a*). Staining of only the cellular margins was observed in cells expressing the mutant CD8 lacking the cytoplasmic tail (Fig. 6 *c*). In these cells, identical staining pattern was observed with or without membrane permeabilization. These data are consistent with localization of CD8 protein on the cell surface with its amino-terminal domain facing outside. Cell-surface expression of the tailless CD8 protein in transfected fibroblasts showed that the cytoplasmic tail of CD8 had no influence on the cellular localization of CD8 protein, or the ability of mAb OKT-8 to recognize epitopes on the extracellular amino-terminal domain of CD8.

In membrane-permeabilized transfectants expressing the chimeric CD8/gp75 TM+Cyt and CD8/gp75 Cyt proteins, the anti-CD8 antibody staining was localized to the

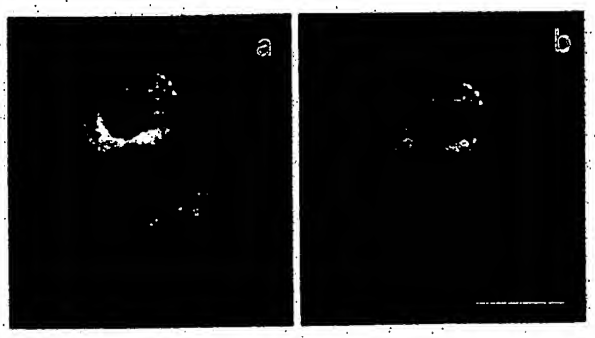

Figure 4. Colocalization of gp75 and LAMP-1 in transfected fibroblasts. Cells fixed in 2% paraformaldehyde and permeabilized with cold methanol were first incubated with rat anti–mouse LAMP-1 antibody (mAb 1D4B), followed by TRITC-conjugated rabbit anti–rat IgG, and finally with FITC-conjugated anti-gp75 antibody (mAb TA99). *a*, gp75 and *b*, LAMP-1. Cells were photographed at th same plane of focus using Optiphot microscope with appropriate optical filters and a barri r filter. Bar, 30 μm.

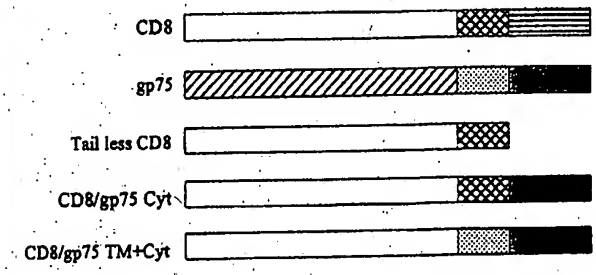

Figure 5. Schematic diagram of wild-type CD8 and gp75 proteins, tailless CD8 mutant, and CD8/gp75 chimeras. CD8 amino-terminal domain, transmembrane region, and the cytoplasmic tail are shown as an open bar, a box with cross hatches, and a box with horizontal lines, respectively. For gp75, the amino-terminal region is shown as hatched bar, transmembrane region as a stippled box, and the cytoplasmic tail as a solid box. CD8 chimeras containing either gp75 cyt plasmic tail alone, or the gp75 transmembrane region and cyt plasmic tail were constructed as described in Materials and Methods.

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juxtanuclear region. There was very little, or no staining of other cytoplasmic structures or the plasma membrane (Fig. 6, *e* and *g*). The pattern of staining suggested localization of the chimeric proteins in the Golgi region and possibly other organelles such as late endosomes and lysosomes present in the Golgi region. The exclusive localization of the chimeric proteins in the Golgi region, and absence of these proteins in peripheral cytoplasmic organelles could be the result of a rapid degradation of the CD8/gp75 chimeras in a proteolytic compartment. Because our studies on the cellular localization of the wild-type gp75 showed that the protein expressed in fibroblasts was localized to LAMP-1–positive vesicles, we tested the possibility that CD8/gp75 chimeras were transported to endosomes/lysosomes where the extracellular domain of CD8 was rapidly degraded by proteases. Mouse L cell fibroblasts were transfected with cDNAs encoding CD8/gp75 chimeras, and the transiently expressed proteins were allowed to accumulate for 24 to 72 h in the presence or absence of the serine protease inhibitor, leupeptin. Cells were fixed and permeabilized at 24, 48, and 72 h, and the cellular distribution of the chimeras was analyzed by immunofluorescence staining with mAb OKT-8. In cells expressing CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeric proteins in the absence of leupeptin, the anti-CD8 antibody staining was localized to the juxtanuclear region (Fig. 6, *e* and *g*), and remained localized to that region even 72 h after transfection (data not shown). When leupeptin was added in the culture medium of transfectants, staining of organelles distributed throughout the cytoplasm, in addition to the juxtanuclear staining, could be seen by 24 h (data not shown) and became prominent by 48 h (Fig. 6, *f* and *h*). Both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras showed identical staining pattern. Leupeptin did not alter the cell-surface localization of the tailless CD8 protein (Fig. 6 *d*). These results allowed us to draw the following conclusions on the sorting of CD8/gp75 chimeras: (*a*) In fibroblasts, both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras were localized in intracellular compartments and failed to accumulate at the cell surface. (*b*) Both chimeras were sorted to a cellular compartment where the extracellular domain of CD8 was rapidly degraded in the absence of protease inhibitors. (*c*) The cytoplasmic tail of gp75, the common domain in both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras, was necessary for intracellular sorting of the CD8 chimeras to a protease-rich compartment, consistent with endosome/lysosome.

Carboxyl–terminal 27 Amino Acids Are Necessary for Intracellular Sorting of gp75

To delineate further the region and amino acid sequences within the gp75 cytoplasmic domain that determine sorting along the endocytic pathway, we generated carboxyl-terminal deletion mutants of gp75. Deletions of varying lengths were generated by Bal I nuclease digestion of linearized gp75 plasmid followed by ligation of a multiple reading frame termination linker.

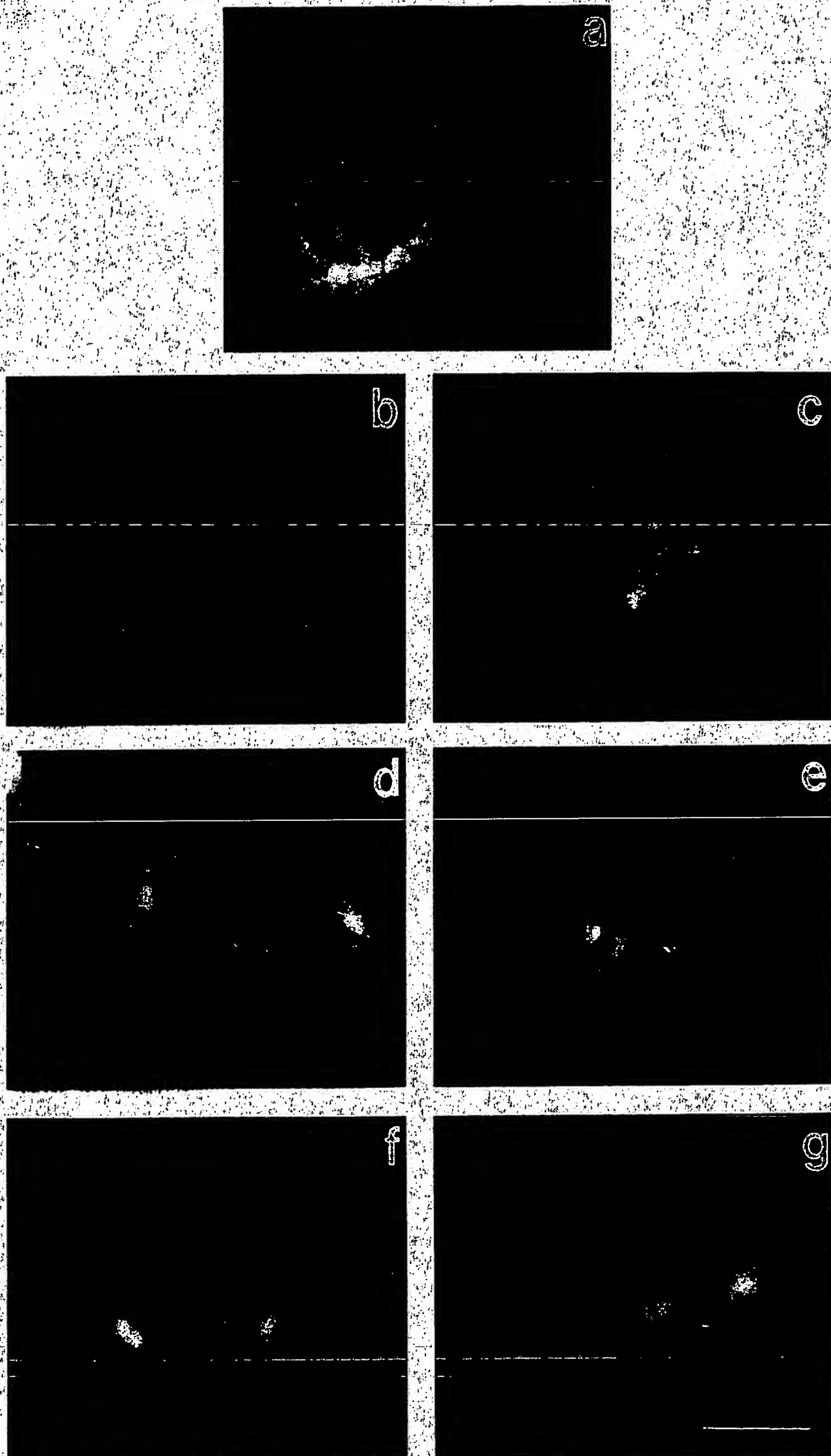
Mouse L cell fibroblasts and a nonpigmented, melanosome-positive and gp75-negative melanoma cell line (SK-MEL-23 clone 22a) were transfected with the carboxyl-terminal deletion mutants of gp75, and the cellular distribution of transiently expressed protein was studied by immunofluorescent staining with the mAb TA99 (Figs. 7 and 8). Transfectants expressing mutant gp75 proteins with deletion of 14 or 20 carboxyl-terminal amino acid residues showed a patchy juxtanuclear staining and punctate cytoplasmic staining (Figs. 7 and 8, *b* and *c*). This pattern of staining was similar to the staining of the wild-type full-length gp75 in transfectants, and also to the distribution of gp75 constitutively expressed in melanoma cells.

Expression of deletion mutants of gp75 lacking the terminal 27 or 34 amino acids in melanoma cells SK-MEL-23 clone 22a and fibroblasts produced a staining pattern that demarcated the cellular boundaries of membrane-permeabilized cells, and a patchy to diffuse intracellular staining (Fig. 7, *f* and *g*; Fig. 8, *d* and *e*), similar to the staining observed in fibroblasts expressing the full-length CD8 protein. Anti-gp75 staining could be seen without membrane permeabilization (Fig. 7, *d* and *e*). These results showed that deletion of up to 20 carboxyl-terminal amino acid residues (ΔC20 construct) did not affect the sorting and intracellular retention of gp75 in melanoma cells. Deletion of an additional seven amino acids, i.e., 27 carboxyl-terminal amino acids (ΔC27 construct), resulted in failure of gp75 to be sorted efficiently for intracellular retention, and the protein appeared on the cell surface. These data demonstrated that amino acid residues between carboxyl-terminal positions 20 and 27 were responsible for intracellular sorting, and suggested that these residues are involved in targeting of gp75 to melanosomes.

Amino Acid Residues between 511 and 517 within the Cytoplasmic Tail of gp75 Constitute a Signal for Retention and Intracellular Sorting of gp75

Carboxyl termini of the deletion mutant CΔ20, which was sorted for retention in intracellular compartments, and the deletion mutant CΔ27, which was expressed on the cell surface, delineated the limits of a 7–amino acid sequence, *asn-gln-pro-leu-leu-thr-asp* (NQPLLTD), that was necessary for the intracellular sorting and targeting of gp75 in both melanocytic cells and fibroblasts (Fig. 9). Substitution of carboxyl-terminal residues of truncated gp75 molecules with amino acid residues encoded by the termination linker did not affect the cellular localization determined

Figure 6. Immunofluorescence microscopy of L cells transfected with wild-type CD8, tailless CD8, and CD8/gp75 chimeric plasmid constructs. Transfected cells expressing wild-type CD8 (*a*), wild-type gp75 (*b*), tailless CD8 (*c* and *d*), CD8/gp75 TM+Cyt (*e* and *f*) or CD8/gp75 Cyt (*g* and *h*) were fixed in 2% paraformaldehyde 48 h after transfection. Cells were stained with anti-CD8 mAb OKT-8 (*a*, *c–h*) or rabbit anti–human gp75 antibody (*b*). Cells in *a*, *b*, and *e–h* were stained after membrane permeabilization, and cells in *c* and *d* were stained without membrane permeabilization. In the absence f th protease inhibitor leupeptin (*e* and *g*), both CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras were localized exclusively to the juxtanuclear Golgi region. (*e* and *g*) White dashes indicate the cellular margins. In the presence f leupeptin (*f* and *h*), staining of distinct cytoplasmic organelles could be seen. Leupeptin had no ffect on the cell-surface expression of the tailless CD8 pr tein (*d*). Bar, 16 μm.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by this sorting signal, as demonstrated by different patterns of distribution of ΔC14, ΔC27, and ΔC34 (Figs. 7 and 8, *b*, *d*, and *e*) mutants containing identical carboxyl-terminal linker residues. These mutants also showed the lack of involvement of additional carboxyl-terminal residues, specifically the glutamine residue at position 518, and the tyrosine residue at position 526, in sorting gp75.

To test whether the NQPLLTD sequence was responsible for intracellular retention of the CD8/gp75 chimeras, we made mutant CD8/gp75 chimeras with the gp75 cytoplasmic tail lacking 20 or 27 carboxyl-terminal amino acids. This was accomplished by introducing a translation stop codon at positions 512 (*gln*) or 518 (*gln*) within the gp75 cytoplasmic tail of the CD8/gp75 TM+Cyt and CD8/gp75 Cyt chimeras. As shown earlier, fibroblasts transfected with the wild-type chimeras stained with anti-CD8 antibody showed characteristic juxtanuclear staining in the absence of the protease inhibitor, leupeptin, and punctate cytoplasmic staining in the presence of leupeptin. Expression of mutant chimeras with the gp75 cytoplasmic tail lacking the carboxyl-terminal 20 amino acids produced a staining pattern identical to the wild-type chimeras (Fig. 10, *a* and *d*). In transfectants expressing the mutant chimeras lacking the 27 carboxyl-terminal amino acids, a staining pattern identical to the staining of the wild-type full-length and tailless CD8 proteins was observed (Fig. 10, *c* and *f*). This staining could be detected without membrane permeabilization, and without the addition of leupeptin during the transient expression, demonstrating the cell-surface localization of the chimeric CD8 protein. These data showed that the peptide sequence NQPLLTD within the cytoplasmic domain of gp75 was also necessary for the intracellular sorting and targeting of the CD8/gp75 chimeras to endosomes/lysosomes in fibroblasts.

QPLLTD Is Conserved among Melanosomal Membrane Proteins

The hexapeptide sequence QPLLTD, consisting of a polar uncharged residue followed by three nonpolar hydrophobic residues, is conserved between both mouse and human gp75 (Fig. 11). Carboxyl-terminal deletion analysis studies showed that the cytoplasmic tail was also necessary for intracellular sorting of mouse gp75 (Xu, Y., S. Vijayasaradhi, and A. N. Houghton, manuscript in preparation). We examined the amino acid sequences of other melanosomal membrane proteins for the presence of similar intracellular sorting signals, including tyrosinase encoded by the *albino* locus, TRP-2 encoded by the *slaty* locus, Pmel 17 encoded by the *silver* locus, P-protein encoded by the *pink-eyed* (*p*) locus, and melanocyte stimulating hormone receptor encoded by the *extension* locus. A conserved peptide sequence QPLLMD is present at a similar position (22 amino acids from the TM domain) in the cytoplasmic tail of both human and mouse tyrosinases. In the carboxyl terminus of Pmel 17 protein, a sequence NSPLLSG is present 36 amino acids downstream from the putative TM domain. Recently, Zhou et al. (1994) have shown that the Pmel 17 is processed to a melanosomal matrix protein. In the human *p*-locus gene product (which is presumably a melanosomal membrane protein with 12 transmembrane domains), a sequence NTPLLR, and in mouse *p*-locus protein, TPLLWN, are present at similar positions within the amino-terminal region. It is important to note that the amino-terminal region of the *p*-locus protein has been predicted to face the cytoplasmic face of the melanosomal membrane (Rinchik et al., 1993). Presence of this conserved sorting signal in the amino-terminal region of the p protein supports both its melanosomal localization, and is consistent with the cytoplasmic orientation of this conserved region in gp75, tyrosinase and Pmel 17. In addition to the residues within the hexapeptide sequence, a glutamic acid located 3–4 residues amino-terminal to the dileucine motif is also conserved among all these melanosomal proteins. No sequences of significant homology to QPLLTD were found in TRP-2, a melanogenic enzyme localized to the melanosome (Fig. 11) and the *e* locus product, a receptor for melanocyte stimulating hormone (sequence not shown).

Discussion

The biogenesis of melanosomes requires intracellular sorting and targeting of melanosomal membrane proteins to appropriate precursor vesicles. This family of glycoproteins include tyrosinase (the *albino* locus product), gp75 (*brown* locus), and Pmel 17 (*silver* locus product). In this study, we have investigated the signals responsible for intracellular sorting of the most abundant melanosomal glycoprotein, gp75. Chimeric CD8 proteins containing the carboxyl tail of gp75, with TM domain of either CD8 or gp75, were retained in the cell and failed to reach the cell surface. This demonstrated that the 36–amino acid carboxyl tail of gp75 was responsible for the intracellular sorting of the chimeric CD8 molecule. Intracellular staining was localized almost exclusively to the juxtanuclear Golgi region after membrane permeabilization. The apparent accumulation of the chimeric CD8 molecules in the Golgi region was not due to retardation of plasma membrane protein transport. When leupeptin was added to the cells after transfection, staining of cytoplasmic vesicles could be seen as early as 24 h, showing that signals in the gp75 carboxyl tail were sufficient to target the chimeric proteins to

Figure 7. Immunofluorescence microscopy of L cell fibroblasts transiently expressing wild-type gp75 (*a*) and carboxyl-terminal deletion mutants of gp75 ΔC14 (*b*), ΔC20 (*c*), ΔC27 (*d* and *f*), or ΔC34 (*e* and *g*). Transfected cells were allowed to express the proteins for 48 h, were fixed in 2% paraformaldehyde, and were stained with mAb TA99 followed by FITC-conjugated anti–mouse IgG with (*a–c*, *f*, and *g*) or without (*d* and *e*) prior membrane permeabilization. In membrane-permeabilized cells, ΔC14 and ΔC20 mutants (*b* and *c*) produced an intracellular staining pattern similar to the full-length wild-type gp75, whereas mutants ΔC27 and ΔC34 (*f* and *g*) showed a distinct staining of cellular margins and a weak intracellular staining. Cells in all panels are shown at identical magnification except in *d* and *e* in which cells are shown at a lower magnification. Bars, 16 μm (*a–c*, *f*, and *g*); 20 μm (*d* and *e*).

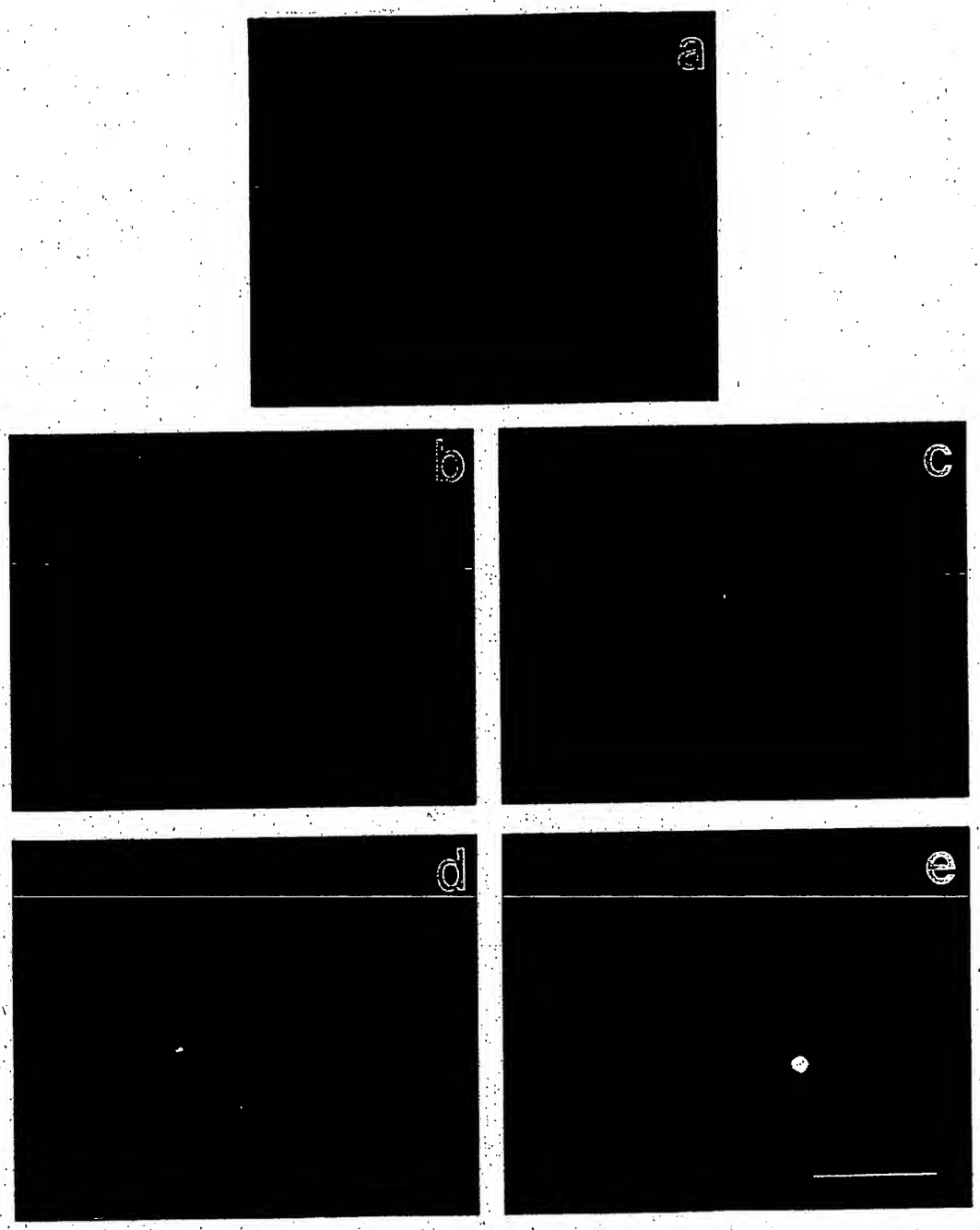

Figure 8. Immunofluorescence microscopy of human melanoma cells (clone 22a) transiently expressing wild-type gp75 (*a*) and carboxyl-terminal deletion mutants of gp75 ΔC14 (*b*), ΔC20 (*c*), ΔC27 (*d*), or ΔC34 (*e*). Transfected cells were allowed to express the proteins for 48 h, and were fixed in 2% paraformaldehyde, permeabilized, and stained with mAb TA99, followed by FITC-conjugated anti-mouse IgG. In membrane permeabilized cells, ΔC14 and ΔC20 mutants (*b* and *c*) produced an intracellular staining pattern similar to the full-length wild-type gp75, whereas mutants ΔC27 and ΔC34 (*d* and *e*) showed a distinct staining of cellular margins and a weak intracellular staining. Bar, 16 μm.

the protease-rich vesicular components of the endosomal/lysosomal pathway. In this proteolytic compartment, the amino-terminal domain of CD8 was rapidly degraded, whereas the highly glycosylated luminal domain of gp75 was stable. Newly synthesized gp75 in fibroblast transfectants has an apparent half-life of 20–24 h, similar to the long half-life of gp75 in melanoma cells (Vijayasaradhi et al., 1991; and Vijayasaradhi, S., and A. N. Houghton, unpublished observations). Sequential carboxyl-terminal deletion mutants allowed us to identify the six-amino acid sequence, Asn-Gln-Pro-Leu-Leu-Thr, critical for the intracellular sorting of gp75 in melanocytic cells and the CD8 chimeras al ng the endocytic pathway.

Upon exit from the TGN, newly synthesized membrane proteins destined for intracellular retention and targeting to organelles are transported to late endosomes. In addi-

wt gp75 ...RARRSMDEANQPLLTDQYQCYAEEYEKLQNPNQSVV

Δ C14 ...RARRSMDEANQPLLTDQYQCY*ALAS*

Δ C20 ...RARRSMDEANQPLLT**DS**

Δ C27 ...RARRSMDE*ALAS*

Δ C34 ...R*ALAS*

Figure 9. Amino acid sequences at the carboxyl termini of wild-type gp75 and deletion mutants. Sequences are shown beginning at the junction of membrane spanning region (TM) predicted from hydrophobicity analysis and the cytoplasmic tail. Amino acid residues are numbered from the amino terminus, beginning with the methionine residue encoded by the initiation codon. Numbers after ΔC represent the gp75 amino acid residues deleted from the carboxyl terminus. Amino acid residues required for the intracellular sorting of gp75 are shown in bold. gp75 terminal amino acids replaced by amino acids encoded the termination linker are shown in italics.

tion to newly synthesized proteins transported from the TGN, late endosomes also receive input from the plasma membrane and extracellular environment. Some cell-surface receptors internalized by endocytosis recycle between early and late endosomes before returning to the plasma membrane or transported to lysosomes for degradation (Trowbridge et al., 1993). Biogenesis of distinct postendosomal compartments such as lysosomes (Peters and von Figura, 1994), transcytotic vesicles, synaptic vesicles (Kelly, 1993), major histocompatibility complex class II containing vesicles (Amigorena et al., 1994; Tulp et al., 1994), and, presumably, melanosomes, requires specific endosomal sorting of intracellular membrane proteins from proteins that recycle between early and late endosomes and plasma membrane.

Intracellular pathways of sorting and targeting of integral membrane proteins to the lysosome have been investigated extensively (Peters and von Figura, 1994). Two distinct signals required for sorting and targeting of lysosomal membrane proteins have been identified. First, carboxyl-terminal deletion studies have shown that a dileucine motif in the cytoplasmic domain of mannose-6-phosphate receptors is necessary for efficient sorting of lysosomal hydrolases from *trans*-Golgi to lysosomes, via late endosomes, by mannose-6-phosphate receptors (Johnson and Kornfeld, 1992). Recently, targeting of LIMP II, a resident lysosomal integral membrane protein, to lysosomes has also been shown to be mediated by a Leu-Ile motif in the cytoplasmic tail (Ogata and Fukuda, 1994; Sandoval et al., 1994). Strikingly, there are two consecutive leucine residues within the

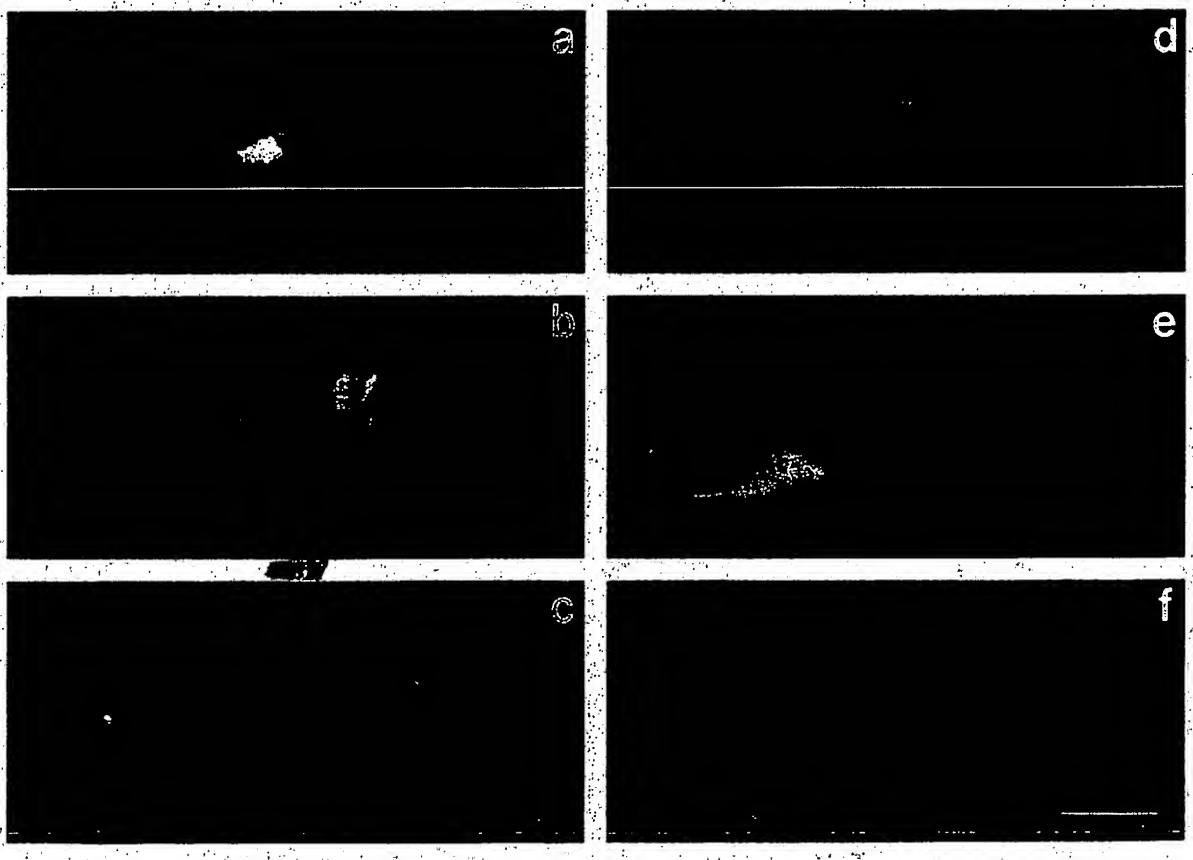

Figure 10. Immunofluorescence microscopy of L cells expressing full-length CD8/gp75 chimeras and carboxyl-terminal deletion mutants of CD8/gp75 chimeras. Cells transfected with CD8/gp75 TM+Cyt (*a*), CD8/gp75 Cyt (*b*), CD8/gp75 TM+Cyt ΔC27 (*c* and *e*), or CD8/gp75 Cyt ΔC27 (*d* and *f*) were cultured f r 48 h with (*a* and *b*) r without (*c–f*) leupeptin in the culture medium. Cells were fixed in 2% paraformaldehyde and stained with mAb OKT-8, followed by FITC-conjugated anti–mouse IgG antibody with (*a–d*) or without (*e* and *f*) pri r membrane permeabilization. Bar, 16 μm.

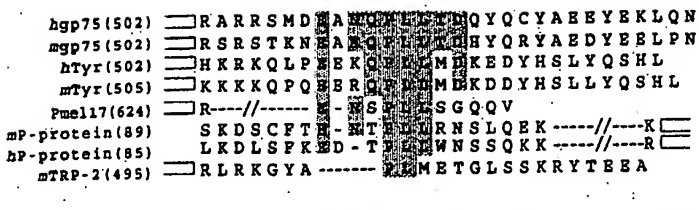

Figure 11. Alignment f carboxyl-terminal amino acid sequences of human (*h*) and mouse (*m*) *brown* locus protein gp75 (TRP-1), *albino* locus protein tyrosinase, *slaty* locus protein dopachrome tautomerase (TRP-2), and the *silver* locus product Pmel 17. Amino acid sequences in single letter code are shown beginning at the junction of membrane spanning region (*open boxes*) and the cytoplasmic tail. For the *pink eyed* locus p protein, the sequences shown are part of the first amino-terminal loop predicted to be facing the cytoplasmic side of the melanosomal membrane. Boxes at the end of the p protein sequences are the first transmembrane regions proximal to the amino-terminal end. Amino acid residues conserved in the region of gp75 sorting signal and the glutamic acid (*E*) amino-terminal to the signal are shown in bold within the stippled box. Single dashes and series of dashes represent breaks introduced between consecutive amino acids for the purpose of sequence alignment. Series of dashes interrupted by back slashes represent stretches of amino acids not shown.

gp75 sorting sequence, Asn-Gln-Pro-Leu-Leu-Thr. The sequence motif Pro-Leu-Leu, located 10–12 amino acids from the TM domain in the cytoplasmic tail, is conserved among human and mouse tyrosinases gp75, and the pMel 17 protein, suggesting that the dileucine sequence in the cytoplasmic-tail sequence is critical for sorting melanosomal proteins along the endocytic pathway.

A close examination of the amino-acid sequences of other melanocyte-specific proteins reveals variations of this notion. First, the *p*-locus protein has multiple potential TM domains, and has been proposed to traverse the melanosomal membrane several times (Gardner et al., 1992). The sequence Pro-Leu-Leu is located in the first amino-terminal loop that faces the cytoplasm. This suggests that the position of the tripeptide sequence with respect to the carboxyl end of the protein is not critical. However, the exact membrane orientation of the *p*-locus protein, and the role of the tripeptide sequence in its intracellular sorting need to be investigated. Second, in TRP-2 (the product of the *slaty* locus, and a type I melanogenic protein like tyrosinase and gp75), only Pro-Leu residues of the tripeptide Pro-Leu-Leu, and not the dileucine motif, is conserved, suggesting the existence of alternative or additional sorting signals. In this context, a second signal, a Gly-Tyr motif, has been shown to be critical for sorting of a family of lysosomal glycoproteins (lgps) in the TGN and targeting them to lysosomes (Williams and Fukuda, 1991; Harter and Mellman, 1992; Matthews et al., 1992). In this family of lgps, the Gly-Tyr signal is located in the cytoplasmic tail at least three amino acids from the carboxyl-terminal residue. It is interesting to note that in TRP-2, a Gly-Tyr motif is present at carboxyl-terminal position 19 and 18 (Jackson et al., 1992; Winder et al., 1993). Therefore, sorting and targeting of TRP-2 may be mediated by this Gly-Tyr motif. This raises the possibility that such additional redundant signals could also be present between amino acid 518 and the COOH terminus in gp75 and other melanosomal proteins.

Although the majority of lysosomal membrane proteins are sorted at the Golgi apparatus for intracellular retention and targeting to lysosomes, some lysosomal proteins may be delivered to lysosomes by an alternative route via endocytosis of proteins transported directly to the cell surface (Harter and Mellman, 1992; Matthews et al., 1992). The tyrosine residue in the *trans*-Golgi sorting signal Gly-Tyr in the cytoplasmic tails of lgps also appears to be critical for internalization of cell-surface lgps, similar to tyrosine signals responsible for receptor-mediated endocytosis. There are multiple tyrosine residues in the cytoplasmic tail of gp75. However, these tyrosine residues do not appear to play a significant role in sorting gp75 along the endocytic pathway by internalization of cell surface gp75, because deletion mutants lacking these tyrosine residues were not detected on the cell surface, but were sorted efficiently to intracellular vesicles.

Some proteins normally present on the cell surface are also known to be targeted to the endosomes/lysosomes under specific physiological or pathological conditions, or when expressed in heterologous cell types. A dileucine motif or a signal containing two adjacent nonaromatic hydrophobic residues is required for the lysosomal targeting of these proteins via endocytic pathway either by *trans*-Golgi sorting along the late endosomal/lysosomal pathway or by internalization from plasma membrane into early endosomes (Letourneur and Klausner, 1992; Pelchen et al., 1992; Aiken et al., 1994; Corvera et al., 1994; Matter et al., 1994; Odorizzi et al., 1994). For example, it has been shown that a dileucine motif in the cytoplasmic tail of CD3γ and CD3δ chains of the T cell surface antigen receptor complex is responsible for the *trans*-Golgi sorting to lysosomes. The same motif is required for the endocytosis and lysosomal degradation of the resident T cell surface protein CD4, induced by human immunodeficiency virus 1 protein nef in T cells. However, newly synthesized CD3 T cell receptor chains, and CD4 are transported to the T cell surface despite the presence of a dileucine motif. This suggests the existence of mechanisms or additional signals that either suppress the dileucine signal during biosynthetic transport or activate the signal for internalization (Letourneur and Klausner, 1992; Pelchen-Matthews et al., 1992). Therefore, it is the context, and not mere presence, of the dileucine motif that appears to determine targeting of proteins to the endosome/lysosome compartment. The fate of proteins entering this pathway then depends, presumably, on sorting events within the endosomal compartment. Although gp75 and lysosomal proteins share sorting signals, in melanocytic cells, additional signals may operate either to target gp75 to distinct melanosomal precursors or to cause retention in endosomes ("modified endosomes") for biogenesis of melanosomes. In this context, it is of interest to note that in addition to the dileucine motif, a glutamic acid located two or three amino acids amino-

terminal to the Pro-Leu-Leu motif is also conserved in all melanocyte-specific proteins, except TRP-2. In the absence of the dileucine signal critical for sorting along endosomal/lysosomal pathway, truncated gp75 containing only the glutamic acid signal is transported to the cell surface. Alternatively, it is possible that additional residues amino-terminal to the Pro-Leu-Leu motif are also critical for intracellular sorting of gp75. These membrane-proximal amino acid residues may play an indirect role by extending the sorting signal into the cytosol for interaction with the protein components of sorting and targeting machinery.

Sorting of gp75 and the CD8/gp75 chimeras along the endosomal/lysosomal pathway has implications for targeting of melanosomal proteins in melanocytic cells and the biogenesis of melanosomes. Sorting of melanosomal proteins along the endosomal/lysosomal pathway could indicate that lysosomes and melanosomes share a common precursor, and that in melanocytic cells melanosomes ("specialized endosomes") evolve from this common precursor. Or, it could indicate biogenesis of two distinct organelles by sorting of proteins along a shared pathway that then diverges to deliver proteins to distinct organelle precursors. Melanosomes and lysosomes have several biochemical characteristics in common with late endosomes. The pH of the lumen of both organelles is acidic (Moellmann et al., 1989; Bhatnagar et al., 1993). The enzyme acid phosphatase, a lysosomal marker, and other hydrolases have been reported to be also present in melanosomes (Seiji and Kikuchi, 1969; Boissy et al., 1987). In mouse melanoma cells, gp75 and LAMP-1 have been shown to colocalize to melanosomal precursors that morphologically resemble endosomes (Zhou et al., 1993). There are potential distinctions in intracellular routes of targeting proteins to these two organelles. The targeting of melanosomal proteins, and the movement of melanosomal membrane proteins appears to be predominantly unidirectional, i.e., after biogenesis of melanosomes, melanin pigment accumulates in the lumen, and eventually the whole organelle is exported into neighboring keratinocytes. Lysosomes receive input from multiple pathways, i.e., in addition to proteins delivered directly from the *trans*-Golgi via endosomes, proteins are also transported from the cell surface to lysosomes for degradation or retention as resident membrane proteins. Lysosomal membrane proteins may also recycle between endosomes and cell surface (Trowbridge et al., 1993).

Identification of the dileucine-containing sequence as a sorting signal in melanosomal proteins, together with the shared features of late endosomes/lysosomes and melanosomes, argues in favor of the notion that melanosomes are specialized endosomes (Zhou et al., 1993). Our data show that the dileucine-containing sequence is critical for sorting and intracellular retention of gp75, indicating that sorting signals and pathways of biogenesis may be shared between melanosomes and lysosomes.

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References

Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono. 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell.* 76:853–864.

Alexander, R. B., W. B. Isaacs, and E. R. Barrack. 1985. Immunogold probes for electron microscopy: evaluation of staining by fluorescence microscopy. *J. Histochem. Cytochem.* 33:995–1000.

Amigorena, S., J. R. Drake, P. Webster, and I. Mellman. 1994. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature (Lond.).* 369:113–120.

Bhatnagar, V., S. Anjaiah, N. Puri, B. N. Darshanam, and A. Ramaiah. 1993. pH of melanosomes of B16 murine melanoma is acidic: its physiological importance in the regulation of melanin biosynthesis. *Arch. Biochem. Biophys.* 307:183–192.

Boissy, R. E., G. E. Moellmann, and R. Halaban. 1987. Tyrosinase and acid phosphatase activities in melanocytes from avian albinos. *J. Invest. Dermatol.* 88:292–300.

Bouchard, B., B. B. Fuller, S. Vijayasaradhi, and A. N. Houghton. 1989. Induction of pigmentation in mouse fibroblasts by expression of human tyrosinase cDNA. *J. Exp. Med.* 169:2029–2042.

Chakraborty, A. K., Y. Mishima, M. Inazu, S. Hatta, and M. Ichihashi. 1989. Melanogenic regulatory factors in coated vesicles from melanoma cells. *J. Invest. Dermatol.* 93:616–620.

Chen, J. W., W. Pan, M. P. D'Souza, and J. T. August. 1985. Lysosome associated membrane protein: characterization of LAMP-1 of macrophage P388 and mouse embryo 3T3 cultured cells. *Arch. Biochem. Biophys.* 239:574–586.

Corvera, S., A. Chawla, R. Chakrabarti, M. Joly, J. Buxton, and M. P. Czech. 1994. A double leucine within the GLUT4 glucose transporter COOH-terminal domain functions as an endocytosis signal. *J. Cell Biol.* 126:979–989.

Foster, M. 1965. Mammalian pigment genet. *Adv. Genet.* 13:311–339.

Gardner, J. M., Y. Nakatsu, Y. Gondo, S. Lee, M. F. Lyon, and M. H. Brilliant. 1992. The mouse *pink-eyed dilution* gene: association with human Prader-Willi and Angelman syndromes. *Science (Wash. DC).* 257:112–114.

Griffiths, G., K. Simon, G. Warren, and K. T. Tokuyasu. 1983. Immunoelectron microscopy using thin, frozen sections: application to studies of the intracellular transport of Semliki Forest virus spike glycoproteins. *In* Methods in Enzymology: Biomembranes. Vol. 96. S. Fleischer and B. Fleischer, editors. Academic Press, New York. 466–485.

Harter, C., and I. Mellman. 1992. Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. *J. Cell Biol.* 117:311–325.

Hearing, V. J., P. Phillips, and M. A. Lutzner. 1973. The fine structure of melanogenesis in coat color mutants of the mouse. *J. Ultrastruct. Res.* 43:88–106.

Jackson, I. J. 1988. A cDNA encoding tyrosinase-related protein maps to the *brown* locus in mice. *Proc. Natl. Acad. Sci. USA.* 85:4392–4396.

Jackson, I. J., D. M. Chambers, K. Tsukamoto, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and V. Hearing. 1992. A second tyrosinase-related protein, TRP-2, maps to and is the mouse *slaty* locus. *EMBO (Eur. Mol. Biol. Org.) J.* 11:527–535.

Jiménez-Cervantes, C., F. Solano, T. Kobayashi, K. Urabe, V. J. Hearing, J. A. Lozano, and J. C. Garcia-Borron. 1994. A new enzymatic function in the melanogenic pathway. The 5, 6-dihydroxyindole-2-carboxylic acid oxidase activity of tyrosinase-related protein-1 (TRP-1). *J. Biol. Chem.* 269:17993–18001.

Johnson, K. F., and S. Kornfeld. 1992. A His-Leu-Leu sequence near the carboxy terminus of the cytoplasmic domain of the cation-dependent mannose-6-phosphate receptor is necessary for the lysosomal enzyme sorting function. *J. Biol. Chem.* 267:17110–17115.

Kelly, R. B. 1993. A question of endosomes. *Nature (Lond.).* 364:487–488.

Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *In* Methods in Enzymology: Recombinant DNA. Vol. 154. R. Wu and L. Grossman, editors. Academic Press, New York. 367–382.

Kwon, B. S., C. Chintamanenei, C. A. Kozak, N. G. Copeland, D. J. Gilbert, N. Jenkins, D. Barton, U. Francke, Y. Kobayashi, and K. K. Kim. 1991. A melanocyte-specific gene, *Pmel* 17, maps near the *silver* coat color locus on mouse chromosome 10 and is in a syntenic region on human chromosome 12. *Proc. Natl. Acad. Sci. USA.* 88:9228–9232.

Letourneur, F., and R. D. Klausner. 1992. A novel di-leucine motif and a tyrosine based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell.* 69:1143–1157.

Margolskee, R. F., P. Kavathas, and P. Berg. 1988. Epstein-Barr virus shuttle vector for stable episomal replication of cDNA expression libraries in human cells. *Mol. Cell. Biol.* 8:2837–2847.

Matter, K., E. Y. Yamamoto, and I. Mellman. 1994. Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL receptor and Fc receptors in MDCK cells. *J. Cell Biol.* 126:991–1004.

Matthews, P. M., J. B. Martinie, and D. M. Fambrough. 1992. The pathway of targeting signal for delivery integral membrane glycoprotein LEP100 to lysosomes. *J. Cell Biol.* 118:1027–1040.

Maul, G. G., and J. A. Brumbaugh. 1971. On the possible function of coated vesicles in melanogenesis of the regenerating fowl feather. *J. Cell Biol.* 48: 41–48.

Moellmann, G., A. Slominski, E. Kuklinska, and A. B. Lerner. 1989. Regula-

tion of melanogenesis in melanocytes. *Pigm. Cell Res.* 1:79–87.
Moyer, F. H. 1963. Genetic effects on melanosome fine structure and ontogeny in normal and malignant cells. *Ann. N.Y. Acad. Sci.* 100:584–606.
Moyer, F. H. 1966. Genetic variations in the fine structure and ontogeny of mouse melanin granules. *Am. Zool.* 6:43–66.
Novikoff, A. B., A. Albala, and L. Biempica. 1968. Ultrastructural and cytochemical observations on B16 and Harding-Passey mouse melanomas: the origin of premelanosome and compound melanosome. *J. Histochem. Cytochem.* 16:299–318.
Odorizzi, C. G., I. S. Trowbridge, L. Xue, C. R. Hopkins, C. D. Davis, and J. F. Collawn. 1994. Sorting signals in the MHC class II invariant chain cytoplasmic tail and transmembrane region determine trafficking to an endocytic processing compartment. *J. Cell Biol.* 126:317–330.
Ogata, S., and M. Fukuda. 1994. Lysosomal targeting of Limp II membrane glycoprotein requires a novel Leu–Ile motif at a particular position in its cytoplasmic tail. *J. Biol. Chem.* 269:5210–5217.
Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagård, and M. Marsh. 1992. The protein tyrosine kinase p^{56lck} inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. *J. Cell Biol.* 117: 279–290.
Peters, C., and K. von Figura. 1994. Biogenesis of lysosomal membrane. *FEBS Lett.* 346:108–114.
Rinchik, E. M., S. J. Bultman, B. Horsthemke, S. T. Lee, R. A. Spritz, K. M. Avidano, M. T. Jong, and R. D. Nicholls. 1993. A gene for the mouse *pink-eyed dilution* locus and for human oculocutaneous albinism. *Nature (Lond.).* 361:72–76.
Rittenhouse, E. 1968. Genetic effects on fine structure and development of pigment granule in mouse hair bulb melanocytes. I. The b and d loci. *Dev. Biol.* 17:351–365.
Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sandoval, I. V., J. J. Arredondo, J. Alcalde, A. G. Noriega, J. Vandekerckhove, M. A. Jimenez, and M. Rico. 1994. The residues Leu(Ile)475-Ile(Leu,Val,-Ala)476, contained in the extended carboxyl cytoplasmic tail, are critical for targeting of the resident lysosomal membrane protein LIMP II to lysosomes. *J. Biol. Chem.* 269:6622–6631.
Seiji, M., and A. Kikuchi. 1969. Acid phosphatase activity in melanosomes. *J. Invest. Dermatol.* 52:212–216.
Shibahara, S., Y. Tomita, T. Sakakura, C. Nager, B. Chaudhuri, and R. Muller. 1986. Cloning and expression of cDNA encoding mouse tyrosinase. *Nucleic Acids Res.* 14:2413–2427.
Silvers, W. K. 1979. The Coat Colors of Mice. A Model for Mammalian Gene Action and Interaction. Springer-Verlag New York Inc., New York. 379 pp.
Smit, J., and W. J. Todd. 1986. Colloidal gold labels for immunocytochemical analysis of microbes. *In* Ultrastructure Techniques for Microorganisms. H. C. Aldrich and W. J. Todd, editors. Plenum Publishing Corp., New York. 469–516.
Tai, T., M. Eisinger, S.-I. Ogata, and K. O. Lloyd. 1983. Glycoproteins as differentiation markers in human malignant melanoma and melanocytes. *Cancer Res.* 43:2773–2779.
Trowbridge, I. S., J. F. Collawn, and C. R. Hopkins. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.* 9:129–161.
Tsukamoto, K., I. J. Jackson, K. Urabe, and P. M. Montague. 1992. A second tyrosinase-related protein, TRP-2, is a melanogenic termed DOPAchrome tautomerase. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:519–526.
Tulp, A., D. Verwoerd, B. Dobberstein, H. L. Poegh, and J. Pieters. 1994. Isolation and characterization of intracellular MHC class II compartment. *Nature (Lond.).* 369:120–126.
Vijayasaradhi, S., B. Bouchard, and A. N. Houghton. 1990. The melanoma antigen gp75 is the human homologue of the mouse *b* (*BROWN*) locus gene product. *J. Exp. Med.* 171:1375–1380.
Vijayasaradhi, S., P. M. Doskoch, and A. N. Houghton. 1991. Biosynthesis and intracellular movement of the melanosomal membrane glycoprotein gp75, the human *b* (*brown*) locus product. *Exp. Cell Res.* 196:233–240.
Williams, M. A., and M. Fukuda. 1991. Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol.* 111:955–966.
Winder, A. J., A. Wittbjer, E. Rosengren, and H. Rorsman. 1993. The mouse *brown* (*b*) locus protein has dopachrome tautomerase activity and is located in lysosomes in transfected fibroblasts. *J. Cell Sci.* 106:153–166.
Zdarsky, E., J. Favor, and I. J. Jackson. 1990. The molecular basis of *brown*, an old mouse mutation, and of an induced revertant to wild type. *Genetics.* 126: 443–449.
Zhou, B.-K., R. E. Boissy, S. Pifko-Hirst, D. J. Moran, and S. J. Orlow. 1993. Lysosome-associated membrane protein-1 (LAMP-1) is the melanocyte vesicular membrane glycoprotein band II. *J. Invest. Dermatol.* 100:110–114.
Zhou, B.-K., T. Kobayashi, P. D. Donatien, D. Bennett, V. J. Hearing, and S. J. Orlow. 1994. Identification of a melanosomal matrix protein encoded by the murine *si(silver)* locus using "organelle scanning". *Proc. Natl. Acad. Sci. USA.* 91:7076–7080.

1: J Cell Biol 1995 Aug;130(4):807-20

Intracellular sorting and targeting of melanosomal membrane proteins: identification of signals for sorting of the human brown locus protein, gp75.

Vijayasaradhi S, Xu Y, Bouchard B, Houghton AN.

Immunology Program, Memorial Sloan-Kettering Cancer Center, New York 10021, USA.

The structural and functional integrity of cytoplasmic organelles is maintained by intracellular mechanisms that sort and target newly synthesized proteins to their appropriate cellular locations. In melanocytic cells, melanin pigment is synthesized in specialized organelles, melanosomes. A family of melanocyte-specific proteins, known as tyrosinase-related proteins that regulate melanin pigment synthesis, is localized to the melanosomal membrane. The human brown locus protein, tyrosinase-related protein-1 or gp75, is the most abundant glycoprotein in melanocytic cells, and is a prototype for melanosomal membrane proteins. To investigate the signals that allow intracellular retention and sorting of glycoprotein (gp)75, we constructed protein chimeras containing the amino-terminal extracellular domain of the T lymphocyte surface protein CD8, and transmembrane and cytoplasmic domains of gp75. In fibroblast transfectants, chimeric CD8 molecules containing the 36-amino acid cytoplasmic domain of gp75 were retained in cytoplasmic organelles. Signals in the gp75 cytoplasmic tail alone, were sufficient for intracellular retention and targeting of the chimeric proteins to the endosomal/lysosomal compartment. Analysis of subcellular localization of carboxy-terminal deletion mutants of gp75 and the CD8/gp75 chimeras showed that deletion of up amino acids from the gp75 carboxyl terminus did not affect intracellular retention and sorting, whereas both gp75 and CD8/gp75 mutants lacking the carboxyl-terminal 27 amino acids were transported to the cell surface. This region contains the amino acid sequence, asn-gln-pro-leu-leu-thr, and this hexapeptide is conserved among other melanosomal proteins. Further evidence showed that this hexapeptide sequence is necessary for intracellular sorting of gp75 in melanocytic cells, and suggested that a signal for sorting melanosomal proteins along the endosomal/lysosomal pathway lies within this sequence. These data provide evidence for common signals for intracellular sorting of melanosomal and

lysosomal proteins, and support the notion that lysosomes and melanosomes share a common endosomal pathway of biogenesis.

PMID: 7642699 [PubMed - indexed for MEDLINE]

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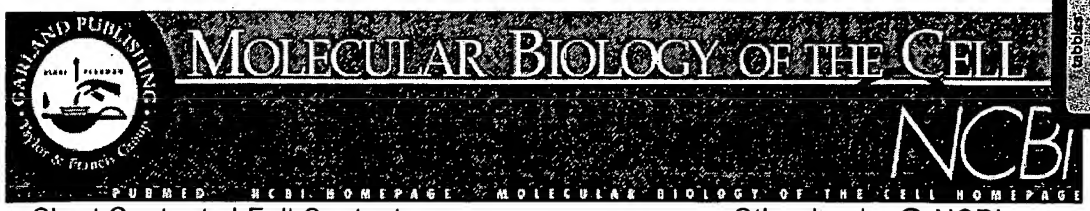

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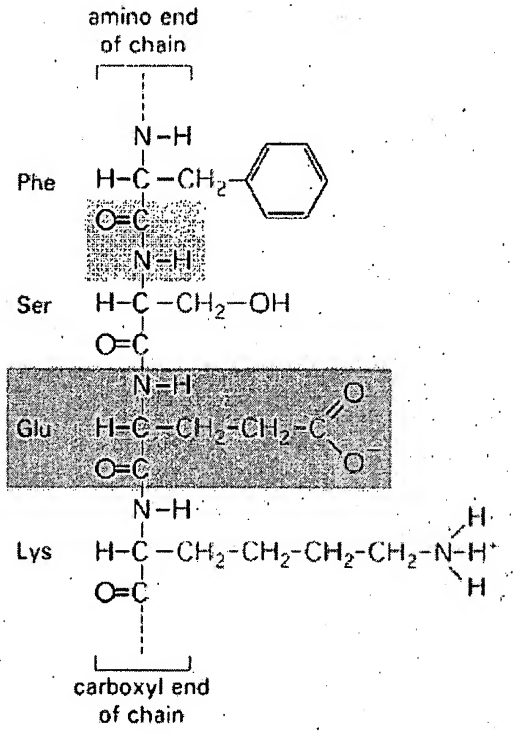

Figure 2-7. A small part of a protein molecule, showing four amino acids. Each amino acid is linked to the next by a covalent *peptide bond,* one of which is shaded *yellow.* A protein is therefore also sometimes referred to as a *polypeptide.* The amino acid *side chains* are shown in *red,* and the atoms of one amino acid (glutamic acid) are outlined by the *gray box.*

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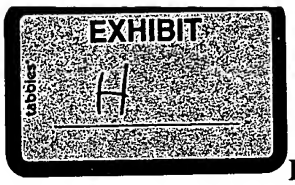

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)
	) Group Art Unit: 1633
Daniel B. Drachman	)
	) Examiner: E. Sorbello
Serial No. 09/205,096	)
	)
Filing Date: December 3, 1998	) Docket No. 01107.77737

For: **TARGETING ANTIGEN-SPECIFIC CELLS FOR SPECIFIC IMMUNOTHERAPY OF AUTOIMMUNE DISEASE**

DECLARATION UNDER RULE 132

I, Daniel B. Drachman, hereby declare:

1. I am the sole inventor of the application referenced above.

2. I have conducted experiments that demonstrate that antigen presenting cells (APCs) transduced by vaccinia virus vectors (VVV) containing genes that encode the influenza hemagglutinin (HA) antigen, Fas ligand (FasL), and truncated FADD cause effective killing of HA-specific T cells in a transgenic mouse.

3. The transgenic mouse used in the experiments has T cells that express an HA-specific T cell receptor. The HA-specific T cell receptor is expressed in approximately 50% of the T cell population of the transgenic mouse.

4. Antigen presenting cells (APCs) specific for HA-specific T cells were prepared by isolating APCs from BALB/c mice and infecting them with an attenuated vaccinia virus vector (VVV). The VVV was genetically engineered to contain three genes encoding: HA (functionally connected to mouse LAMP1), Fas ligand, and truncated FADD. Control APCs were prepared by infecting APCs with an attenuated VVV encoding two genes: Fas ligand and truncated FADD gene. The control APCs thus did not contain the gene encoding HA.

5. An HA transgenic mouse as described in paragraph 3 was injected intraperitoneally

(IP) with $5.0x10^7$ APCs specific for HA-specific T cells as described in paragraph 4. A second HA transgenic mouse was injected IP with $5.0x10^7$ of the control APCs described in paragraph 4. Two HA transgenic mice served as untreated controls and did not receive an injection of APCs.

6. HA-specific T cell killing was measured in each of the HA transgenic mice by flow cytometry. Peripheral blood lymphocytes were collected from the HA transgenic mice at two, five, and eight days after APC injections. The percentage of HA-specific $CD4^+$ T cells in the total $CD4^+$ T cell population of the mice was determined.

7. The percentage of HA-specific $CD4^+$ T cells in the mouse injected with APCs specific for HA-specific T cells was greatly reduced two days following injection compared to the mouse injected with the control APCs or untreated control mice. Specifically, the percentage of HA-specific $CD4^+$ T cells was sharply reduced to 9.4% compared to 59.2% in the mouse injected with control APCs, or 48.8% in the untreated control mice. At 5 and 8 days post APC injection the percentage of HA-specific $CD4^+$ T cells rebounded somewhat but remained reduced compared to the mouse that received the control APCs or the untreated control mice. See Table 1.

Table 1

APCs injected into mouse that express	% $CD4^+$ T cells expressing the HA receptor at 2 days	% $CD4^+$ T cells expressing the HA receptor at 5 days	% $CD4^+$ T cells expressing the HA receptor at 8 days
HA-LAMP-sig + FasL +trFADD	9.4	20.9	24.5
FasL + FADD	59.2	40.8	39.1
No APCs injected	48.8	48.8	48.8

8. Proliferation of HA-specific T cells in the transgenic mice was also assessed. The mice were euthanized twelve days after injection. Lymph node cells and splenocytes were obtained from the euthanized mice and were stimulated with HA *in vitro*. Proliferation was measured by determining the amount of radioactivity incorporated into cells one day after pulsing with ^{3}H-TdR (tritiated deoxyribothymidine).

9. We found that proliferation of HA-specific splenocytes was reduced by 3.0-3.6 fold in

the HA transgenic mouse injected with APCs specific for HA-specific T cells compared to the mouse that received the control APCs or the two untreated control mice. Similarly, proliferation of HA-specific lymph node cells was approximately 2.4-2.7 fold lower in the HA transgenic mouse injected with APCs specific for HA specific T cells compared to the transgenic mouse injected with control APCs or two untreated control HA transgenic mice. See Table 2.

Table 2

APCs that express	Counts measured in splenocytes ($x10^3$)	Counts measured in lymph node cells ($x10^3$)
HA-LAMP-sig + FasL +trFADD	34.8	48.6
FasL + FADD	106.5	129.9
No APCs injected	125.9	118.3

10. The flow cytometry and cell proliferation measurements are consistent with and support the claimed method of using APCs specific for a particular antigen-specific T cell population to activate those particular antigen-specific T cells. Co-administration of a product which is detrimental to activated T cell proliferation leads to a reduction in the antigen-specific $CD4^+$ T cell population and leads to a reduction in antigen-stimulatable proliferation of splenocytes and lymphocytes.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that false statements made willfully are punishable by fine, imprisonment, or both a fine and imprisonment under Section 1001 of Title 18 of the United States; and further that false statements made willfully may jeopardize the validity of any patent issuing on an application in which the false statements were made.

1/3/02
Date

Daniel B. Drachman

Daniel B. Drachman

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1: Cancer Res 1996 Dec 15;56(24):5672-7 Related Articles, Links

Retroviral transduction of human dendritic cells with a tumor-associated antigen gene.

Reeves ME, Royal RE, Lam JS, Rosenberg SA, Hwu P.

Surgery Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892, USA.

Dendritic cells (DCs) are potent antigen-presenting cells that can activate quiescent T lymphocytes. When pulsed with tumor-associated antigen (TAA) peptide or protein, murine DCs can provide antitumor immunity. We reasoned that DCs retrovirally transduced with TAA genes might have important advantages over peptide- or protein-pulsed DCs, including long-term TAA presentation in vivo, and presentation of important but undefined epitopes. Therefore, we attempted to retrovirally transduce human DCs with a melanoma TAA gene (MART-1) and determine whether these transduced DCs could raise a specific antitumor response from quiescent autologous T lymphocytes. After retroviral transduction, human CD34+ cells were differentiated into DCs in vitro using granulocyte macrophage colony-stimulating factor, tumor necrosis factor alpha, and stem cell factor. This method consistently yielded a population of DCs as analyzed by morphology, phenotype, and MLR. Flow cytometric analysis revealed that 22-28% of cells expressing the DC phenotype also expressed a transduced marker gene. When DCs were transduced with the gene encoding MART-1, they stimulated much higher levels of cytokine release by MART-1-specific tumor-infiltrating lymphocytes than control DCs transduced with an irrelevant gene. In vitro stimulation using MART-1-transduced DCs but not control-transduced DCs raised specific antitumor CTLs from autologous quiescent T cells. These results provide evidence that human DCs can be retrovirally transduced with a TAA gene and that these transduced cells can raise a specific antitumor immune response in vitro. Transduced DCs may be useful for in vivo immunization against TAA.

PMID: 8971174 [PubMed - indexed for MEDLINE]

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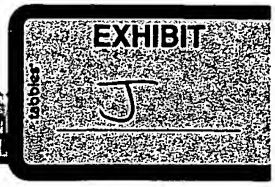

1: Hum Gene Ther 1997 Jul 20;8(11):1355-63 Related Articles, Links

Dendritic cells transduced with an adenoviral vector encoding a model tumor-associated antigen for tumor vaccination.

Wan Y, Bramson J, Carter R, Graham F, Gauldie J.

Department of Pathology, McMaster University, Hamilton, Ontario, Canada.

Evaluation of the potential role of dendritic cells (DCs) as adjuvants for tumor vaccination has focused primarily on techniques that load DCs with peptide tumor antigens. Our aim has been to optimize the induction of antitumor immunity by enhancing the ability of DCs to present tumor-associated antigens endogenously to the afferent lymphatic system in the appropriate major histocompatibility complex (MHC)-restricted context. We have used replication-defective adenovirus vectors (Ads) to transduce DCs with various genes, including tumor antigen genes. We found that 90% of murine bone marrow derived-DCs could be infected with an Ad vector expressing the beta-galactosidase gene and still retain their physiologic and phenotypic characteristics. Furthermore, we demonstrated that transgene expression was detectable in the spleen for at least 3 days following intravenous injection of Ad-transduced DCs. Using a polyoma middle T (PymT) transgenic murine mammary carcinoma model, we have shown that a single injection of 10(5)-4 x 10(6) DCs transduced with an Ad vector expressing PymT provided complete and specific protection against tumor cell challenge in 100% of vaccinated animals. Immunization against the PymT tumor by injection with the PymT expressing Ad vector alone resulted in varying degrees of effectiveness, was highly dependent upon the route of administration, and led to significant hepatic toxicity that was not seen in mice immunized with DC transduced with the Ad vector. Our results suggest that: (i) DCs can be very efficiently modified by ex vivo Ad transduction to express tumor-specific antigens, (ii) such modified DCs appear nontoxic and stimulate a potent antitumor response.

PMID: 9295130 [PubMed - indexed for MEDLINE]

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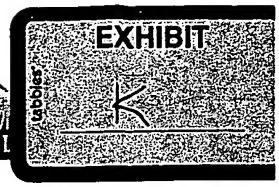

1: Hum Gene Ther 1997 Sep 20;8(14):1651-8

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Efficient retrovirus-mediated gene transfer of dendritic cells generated from CD34+ cord blood cells under serum-free conditions.

Bello-Fernandez C, Matyash M, Strobl H, Pickl WF, Majdic O, Lyman SD, Knapp W.

Vienna International Research Cooperation Center at Novartis Forschungsinstitut, University of Vienna, Austria.

A retroviral-vector encoding the low affinity nerve growth factor receptor (LNGFR) was used to transduce dendritic cells (DCs) generated from CD34+ cord blood (CB) progenitor cells under serum-free conditions. Transduction efficiency was monitored by flow cytometry (FACS) using a specific monoclonal antibody. Prior to retroviral infections, CD34+ CB cells were stimulated for 60 h in a serum-free medium containing a DC differentiation inducing cytokine cocktail: stem cell factor (SCF), granulocyte/macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNFalpha), and transforming growth factor beta 1 (TGF-beta1). Addition of flt3-ligand (FL) to the aforementioned growth factors significantly enhanced cell expansion (41.7+/-11.5 fold vs. 22.5+/-4.7 fold without FL) and generation of CD1a+ DCs (mean 45.7+/-9.8% vs. 28+/-6.5% without FL, n = 4,p = 0.01). Furthermore, FL significantly increased the proportion of CD1a+LNGFR+ cells (mean 10%+/-4.4% vs. 6%+/-2.4 without FL n = 4, p = 0.03). When serum-free viral supernatants were used to infect DCs progenitors under entirely serum-free conditions and with the most potent cytokine combination, approximately one-third of the CD1a+ DCs generated co-expressed the LNGFR gene. Moreover, the transduced gene was also identified in more mature CD1a+CD80+ and CD1a+CD86+ DCs after 12-14 days of culture. In addition, transduced CD1a+ DCs maintained their functional properties, stimulating allogeneic T cells with similar efficiency as nontransduced CD1a+ DCs. Thus, the serum-free system described allows efficient generation and transduction of CD1a+ DCs derived from CD34+ progenitor cells and may be very useful for future therapeutic applications of DCs.

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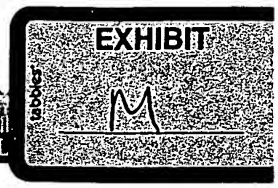
Rapid purification of human Langerhans cells using paramagnetic microbeads.

Simon JC, Dittmar HC, de Roche R, Wilting J, Christ B, Schopf E.

Department of Dermatology, University Freiburg, Germany.

Detailed studies on the biology of Langerhans cells (LC), which account for only 1-3% of all epidermal cells, require isolation from their cutaneous symbionts. Several techniques of LC isolation have been reported, including positive enrichment with mAb coupled to immunomagnetic beads. The disadvantage of this technique is the size of the beads (approximately 2-5 microns), which can interfere with subsequent phenotypic and functional analyses. This limitation prompted us to test whether paramagnetic microbeads (15 nm) employed by the MACS system could be used to purify LC from human skin. To isolate fresh LC (fLC), epidermal cell suspensions (EC) were stained with anti-CD1a mAb and with appropriate secondary reagents conjugated to microbeads and to FITC. They were then passed over a separation column and exposed to a strong magnetic field. Thereafter both CD1a-depleted and CD1a-enriched cells were collected. Cultured LC (cLC) were isolated by staining 72-h cultured EC with anti-HLA-DR mAb followed by the same isolation procedure. Using this technique, we could routinely isolate viable EC that were 45-88% CD1a+ or HLA-DR+ as determined by FACS. Two-color FACS analysis demonstrated the majority of MACS-purified cells to be CD1a+/HLA-DR+, indicating that they were indeed LC. By transmission electron microscopy (TEM), the MACS-purified CD1a+/HLA-DR+ cells showed typical ultrastructural characteristics of LC. Furthermore, MACS-purified fLC or cLC were functionally intact, because they stimulated the proliferation of alloreactive T cells in a primary, one-way, mixed epidermal cell leukocyte reaction (MECLR). We conclude that MACS-separation is an efficient and rapid method to isolate human fLC and cLC of high purity and unimpaired function.

PMID: 7551563 [PubMed - indexed for MEDLINE]



A method for the rapid isolation of human epidermal Langerhans cells using immunomagnetic microspheres.

Hanau D, Schmitt DA, Fabre M, Cazenave JP.

INSERM, Universite Louis Pasteur, Strasbourg, France.

Because Langerhans and indeterminate cells are the only epidermal cells that express the specific CD1a surface antigen T6, we have used immunomagnetic monodisperse polymer microspheres for positive selection of human epidermal Langerhans and indeterminate cells. Epidermal cells in suspension are successively incubated with a murine monoclonal anti-T6 antibody of the IgG1 subclass and then with magnetic beads coated with a sheep anti-mouse IgG1. Rosetted cells are obtained and then easily separated from the non-rosetted cells using a magnet. The two cell fractions are characterized by phase contrast microscopy, immunofluorescence, electron microscopy, and the skin cell-lymphocyte reaction. All the rosetted cells (1.5 to 5% of the total epidermal cells) express T6 antigen by indirect immunofluorescence and under the electron microscope possess all the ultrastructural characteristics of Langerhans cells. Moreover, the rosetted Langerhans cells remain functional: Under the electron microscope they internalize by receptor-mediated endocytosis gold labeled anti-T6 antibody, and in the skin cell-lymphocyte reaction they stimulate allogeneic lymphocytes. In contrast, the rosette depleted cell fraction is deprived of T6 positive cells and unable to stimulate allogeneic lymphocytes. The immunomagnetic depletion of epidermal cells is a simple and rapid method to isolate functional human Langerhans cells with good yield and high purity (97%). This technique should be of value in the study of the pharmacology of Langerhans cells and in the investigation of the interactions of Langerhans cells with keratinocytes or lymphocytes.

PMID: 3411146 [PubMed - indexed for MEDLINE]

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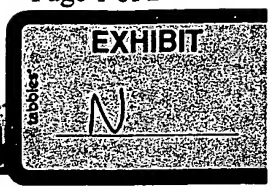

A method for the isolation and serial propagation of keratinocytes, endothelial cells, and fibroblasts from a single punch biopsy of human skin.

Normand J, Karasek MA.

Department of Dermatology, Stanford University School of Medicine, California 94305, USA.

When multiple types of cells from normal and diseased human skin are required, techniques to isolate cells from small skin biopsies would facilitate experimental studies. The purpose of this investigation was to develop a method for the isolation and propagation of three major cell types (keratinocytes, microvascular endothelial cells, and fibroblasts) from a 4-mm punch biopsy of human skin. To isolate and propagate keratinocytes from a punch biopsy, the epidermis was separated from the dermis by treatment with dispase. Keratinocytes were dissociated from the epidermis by trypsin and plated on a collagen-coated tissue culture petri dish. A combination of two commercial media (Serum-Free Medium and Medium 154) provided optimal growth conditions. To isolate and propagate microvascular endothelial cells from the dermis, cells were released following dispase incubation and plated on a gelatin-coated tissue culture dish. Supplementation of a standard growth medium with a medium conditioned by mouse 3T3 cells was required for the establishment and growth of these cells. Epithelioid endothelial cells were separated from spindle-shaped endothelial cells and from dendritic cells by selective attachment to Ulex europeus agglutinin I-coated paramagnetic beads. To establish fibroblasts, dermal explants depleted of keratinocytes and endothelial cells were attached to plastic by centrifugation, and fibroblasts were obtained by explant culture and grown in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS). Using these isolation methods and growth conditions, two confluent T-75 flasks of keratinocytes, one confluent T-25 flask of purified endothelial cells, and one confluent T-25 flask of fibroblasts could be routinely obtained from a 4-mm punch biopsy of human skin.(ABSTRACT TRUNCATED AT 250 WORDS)

PMID: 8589888 [PubMed - indexed for MEDLINE]

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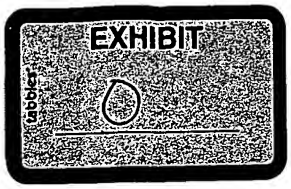

1: J Invest Dermatol 1983 Jul;81(1 Suppl):24s-8s Related Articles, Links

Culture of human keratinocytes in liquid medium.

Karasek MA.

Since 1967, major advances have been made in procedures to isolate and maintain keratinocytes in liquid medium. Human keratinocytes and those from several laboratory animal species may now be isolated from skin either by direct trypsinization of minces, from split-thickness skin following the separation of the epidermis from the dermis, or from the outgrowth from tissue explanted in liquid medium. Isolated keratinocytes display several distinct stages leading to terminal maturation. These include attachment, spreading, reassociation, multiplication, and maturation. Conditions under which each of these stages can be blocked are known and thus provide an opportunity to observe and characterize the biochemical and morphologic changes at each stage of maturation. Although keratinocytes in liquid simulate many of the typical and important characteristics observed in these cells in vivo, the conditions required to reproduce other important functions of keratinocytes have not yet been defined. These functions include the synthesis of basement membranes, lamellar bodies, and keratohyaline granules and the appropriate alignment of lipids and proteins in the completely keratinized cell.

Publication Types:
- Review

PMID: 6306116 [PubMed - indexed for MEDLINE]

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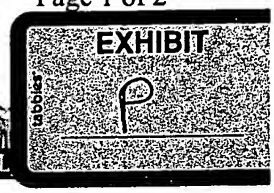

1: Clin Exp Immunol 1994 Nov;98(2):330-6 Related Articles, Links

Isolation and characterization of migratory human skin dendritic cells.

Richters CD, Hoekstra MJ, van Baare J, Du Pont JS, Hoefsmit EC, Kamperdijk EW.

Department of Cell Biology, Faculty of Medicine, Vrije Universiteit, Amsterdam, The Netherlands.

A method is described to isolate and characterize human skin dendritic cells (DC). This method is based on the migratory capacities of these cells. The cells migrated 'spontaneously' out of split-skin explants into the medium during a 24-h culture period and contained up to 75% CD1a+ cells. After removal of co-migrated T cells and macrophages, the highly enriched (> 95% CD1a+) DC showed potent allo-antigen-presenting capacities. About 25% of the CD1a+ cells were also positive for the dermal DC marker CD1b, whereas only 15-20% of the cells contained Birbeck granules, the characteristic cell organelle of the epidermal Langerhans cell. Before culture, CD1a+ DC were observed on cryostat sections not only in the epidermis but also in the dermis. After culture, the number of CD1a+ cells in both epidermis and dermis had decreased. Not all the cells had migrated during the culture period; some CD1a+ cells could still be detected in the epidermis and dermis after culture. Thus, using this method, potent allo-stimulating CD1a+ cells, migrating from both epidermis and dermis, can be obtained without the use of enzymes.

PMID: 7955541 [PubMed - indexed for MEDLINE]

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CD40-activated Human B Cells: An Alternative Source of Highly Efficient Antigen Presenting Cells to Generate Autologous Antigen-specific T Cells for Adoptive Immunotherapy

Joachim L. Schultze,* Sabin Michalak,* Mark J. Seamon,* Glennon Dranoff,* Karin Jung,* Johnathan Daley,* Julie C. Delgado,‡ John G. Gribben,* and Lee M. Nadler*

*Department of Adult Oncology, Dana-Farber Cancer Institute, Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School, and the ‡Division of Immunogenetics, Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Abstract

Multiple clinical trials have shown the efficacy of adoptively transferred allogeneic antigen-specific T cells for the treatment of viral infections and relapsed hematologic malignancies. In contrast, the therapeutic potential of autologous antigen-specific T cells has yet to be established since it has been technically difficult to generate sufficient numbers of these T cells, ex vivo. A major obstacle to the success of this objective derives from our inability to simply and rapidly isolate and/or expand large numbers of highly efficient antigen presenting cells (APCs) for repetitive stimulations of antigen-specific T cells in vitro. We show that autologous CD40-activated B cells represent a readily available source of highly efficient APC that appear to have several important advantages over other APCs for ex vivo T cell expansion including: (*a*) methodological simplicity necessary to generate continuously large numbers of APCs from just 50 cm^3 of peripheral blood without loss of APC function; (*b*) capacity to induce high peak T cell proliferation and interferon-γ production without IL-10 production; (*c*) ease in cryopreservation; and (*d*) markedly reduced cost. We, therefore, contend that CD40-activated B cells are an alternative source of highly efficient APCs with which to generate antigen-specific T cells ex vivo for autologous adoptive immunotherapy. (*J. Clin. Invest.* 1997. 100:2757–2765.) Key words: B lymphocytes • dendritic cells • antigens, CD40 • immunotherapy, adoptive • T cell proliferation

Introduction

Numerous unique peptide antigens have been identified that induce T cell specific immunity to pathogens (1–3) and tumor cells (4). Induction of productive T cell immunity requires efficient presentation of peptide antigens by professional antigen-presenting cells (APCs).[1] Although dendritic cells (DCs) (5–12), activated macrophages (13), or activated B cells (14–22) are all capable of presenting peptides, DCs are considered to be the most efficient APC since fewer DCs are required to induce an optimal T cell immune response (8). In addition to their capacity to present antigen, DCs are also highly efficient at antigen capture, processing, and migration (reviewed in reference 23). Therefore, DCs have been selected as the "APC of choice" to generate antigen-specific T cells for immunotherapy (5, 6, 24, 25).

Since DCs constitute only 0.1–0.5% of human peripheral blood (PB) mononuclear cells, considerable difficulty and expense has been experienced in obtaining sufficient numbers of highly enriched mature DCs (26). Cytokines like GM-CSF and IL-4 have permitted selection and ex vivo expansion of functional DCs (7, 27–29). However, by 2–3 wk, DCs cease to proliferate and become less efficient at presenting antigen. Therefore, to obtain fresh DCs with which to repetitively stimulate autologous T cells, multiple phlebotomies would be required. Alternatively DCs could be generated from $CD34^+$ enriched progenitors (30, 31). Indeed, significantly larger numbers of DCs have been generated from this source (32, 33). However, isolation of DC precursors will likely require either chemotherapy or cytokine pretreatment, leukophereses, and $CD34^+$ stem cell collection (33). Moreover, since mature DCs have yet to be successfully cryopreserved, repetitive generation of DCs either from cryopreserved precursors or from fresh DC sources will be required. Although potentially sufficient numbers of DCs might be generated using any of the above technologies (26), the complexity and cost of preparation of DC precursors and generation of functional DCs limits their utility as APCs for the ex vivo generation of antigen specific T cells. To overcome these obstacles, we sought an alternative, cost-effective source of autologous APCs that could be simply generated from small quantities of human PB, which would result in large numbers of APCs to present peptide antigen efficiently to T cells. Here, we show that CD40-activated peripheral blood B cells (CD40-Bs) fulfill these criteria. Moreover, through comparison with DCs, we have identified several unique characteristics of CD40-Bs that suggest that they might be the optimal APC with which to generate antigen-specific T cells ex vivo for adoptive immunotherapy.

Methods

Donors and cell lines. All specimens were obtained following approval by the institutional Scientific Review Committee. Informed consent for blood donations was obtained from all volunteers. Short-term cultured melanoma cell lines were generated from biopsies of two HLA-A*0201$^+$ and two HLA-A*0201$^-$ patients with melanoma. Human PBMC from healthy donors were obtained by phlebotomy or leukopheresis followed by Ficoll-density centrifugation.

The CD40 ligand system for the culture of normal human PB B

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; CD40-B, CD40-activated B cell; CD40L, CD40 ligand; DC, dendritic cell; MLR, mixed lymphocyte reaction.

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cells. B cells from PBMC were stimulated via CD40 using NIH3T3 cells transfected by electroporation with the human CD40 ligand (t-CD40L cells) (19). The expression of the human CD40 ligand on the transfectants has been stable over a period of > 3 yr. Phenotypic analysis have been performed regularly on these cells and in all analyses > 95% of t-CD40L cells were positive for human CD40 ligand with a mean intensity of fluorescence (MIF) between 80- and 300-fold over background (MIF = 10). t-CD40L cells were negative for human MHC class I and II antigens and therefore no targets for human $CD4^+$ or $CD8^+$ T cells. t-CD40L cells were also tested for all murine viruses known and found to be negative. For B cell cultures, t-CD40L cells were lethally irradiated (96Gy) and subsequently plated on 6-well plates (Costar, Cambridge, MA) at a concentration of 0.4×10^5 cells/well in medium containing 40% DME (Gibco/BRL, Gaithersburg, MD), 40% F12 (Gibco/BRL) 10% FCS, 2 mM glutamine (Gibco/BRL), and 15 μg/ml gentamicin (Gibco/BRL). After an overnight culture at 37°C in 5% CO_2 t-CD40L cells were adherent and could be used for coculture. Before adding PBMC, t-CD40L cells were washed twice by rinsing the plates with PBS. CD40-Bs were generated from PBMC by simply coculturing whole PBMC at 2×10^6 cells/ml with t-CD40L in the presence of IL-4 (2 ng/ml; Immunex, Seattle, WA) (19, 34–37) and cyclosporin A (CsA) at 5.5×10^{-7} M in Iscove's MDM (Gibco/BRL) supplemented with 10% human AB serum, 50 μg/ml transferrin (Boehringer Mannheim, Indianapolis, IN), 5 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), and 15 μg/ml gentamicin (Gibco/BRL) at 37°C in 5% CO_2. The concentration of CsA used in the culture system was found to suppress T cell proliferation without affecting B cell growth. Cultured cells were transferred to new plates with fresh irradiated t-CD40L cells every 3–5 d. Once the cultured PBMC were > 75% $CD19^+$ they were cultured at concentrations of $0.75–1.0 \times 10^6$ cells/ml. If cells were used for analysis or cryopreserved, only a small proportion of cells was recultured and the potential total increase was then calculated. Total number of viable cells was assessed by trypan blue exclusion and the number of $CD19^+$ B cells by immunophenotypic analysis on days 0, 5, 8, and weekly thereafter (37). Before use in functional assays, CD40-Bs were always Ficoll-density centrifuged followed by washing with PBS twice to remove nonviable cells including remaining t-CD40L cells.

Dendritic cell preparation and culture. PBMC were depleted of T, B, and natural killer (NK) cells by magnetic bead depletion (19, 37). DCs were generated from the remaining cell fraction (1.3×10^6 cells/ml, > 80% $CD14^+$) with GM-CSF (50 ng/ml, Genzyme, Cambridge, MA) and IL-4 (10 ng/ml, Immunex, Seattle, WA) in Iscove's MDM (Gibco/BRL) supplemented with 5% human AB serum, 50 μg/ml transferrin (Boehringer Mannheim), 5 μg/ml insulin (Sigma Chemical Co.), 2 mM glutamine (Gibco/BRL), and 15 μg/ml gentamicin (Gibco/BRL) at 37°C in 5% CO_2. Cytokines were added at the beginning of culture and every third day thereafter. To determine maximum expansion DCs were cultured up to 30 d with GM-CSF and IL-4. For functional analysis DCs were maturated on day 6 for 48 h with either TNF-α (30 ng/ml; Genyzme) or t-CD40L before use as APCs in allogenic mixed lymphocyte reaction (allo-MLRs).

T cells. Whole $CD3^+$ T cells and T cell subsets ($CD4^+$, $CD4^+$ $CD45RA^+$ $CD45RO^-$, $CD4^+$ $CD45RO^+$ $CD45RA^-$, $CD8^+$) were obtained from PBMC by magnetic bead depletion of non–T cells (19, 37). Preparations were always > 97% as assessed by immunophenotypic analysis.

Immunofluorescence studies. Dual-color FACS® analysis using directly conjugated mAbs (19, 37) was performed to determine the surface expression of CD1a (T6), CD3 (T3), CD4 (T4), CD8 (T8), CD14 (My4), CD19 (B4), CD20 (B1), CD23 (B6), CD33 (My9), CD45RA (2H4), CD45RO (UCHL1), CD56 (NKH1), CD83 (HB15), MHC class I (B9.12.1) and II (I3) (Coulter Inc., Miami, FL), CD54 (Leu-54; Becton Dickinson, San Jose, CA), CD58 (Amac, MA), CD80 (C4; Repligen Inc., Cambridge, MA), CD86 (IT2.2; PharMingen, San Diego, CA). To determine the source of IFN-γ and IL-10 in cocultures these cytokines were detected by intracellular staining using anti–IFN-γ and anti–IL-10 mAbs (PharMingen) and the Caltag Fix and Perm Kit (Caltag, Burlingame, CA) for intracellular staining.

Peptides. The tyrosinase peptide YMNGTMSQV (369–377) and the influenza A matrix peptide GILGFVFTL (58–66) were synthesized (38) by the Dana-Farber molecular core facility.

Allo-MLR. Allogeneic whole $CD3^+$ T cells or T cell subsets from healthy individuals were plated at 1×10^5 T cells/well with 10^1 to 10^5 irradiated (32 Gy) DCs or CD40-Bs/well in RPMI-1640 supplemented with 5% human AB serum (Sigma Chemical Co.), 2 mM glutamine (Gibco/BRL), 15 μg/ml gentamicin (Gibco/BRL) (RPMI-5) (19). Determination of [^{3}H]thymidine incorporation was performed in triplicates on days 2 to 8.

ELISA for interferon-γ and IL-10. Interferon-γ or IL-10 were detected by ELISA (Endogen, Woburn, MA) in supernatants of cultures of allogeneic T cells either stimulated with CD40-Bs or DCs. Supernatants were collected after 3–8 d to determine peak cytokine accumulation.

Reverse transcriptase (RT-PCR) for tyrosinase. Tyrosinase in melanoma cells was detected by RT-PCR using previously published primers (39).

Immunocytology. Immunocytology for latent membrane protein (LMP-1) and Epstein-Barr virus nuclear antigen-2 (EBNA-2) on cytospin preparations of CD40-Bs was performed as previously described (40).

In vitro CTL response induction. CD40-Bs of HLA-A*0201$^+$ donors were loaded with peptide (50 μg/ml) in the presence of human β2-microglobulin (3 μg/ml; Sigma Chemical Co.), irradiated (32 Gy) and added to purified $CD8^+$ T cells (> 98%) of the same donor at a ratio of T:CD40-Bs = 4:1 in RPMI-5 containing IL-7 (10 ng/ml; Genzyme) (38). At day 7, T cell cultures were harvested, Ficoll density centrifuged to remove nonviable cells, washed twice, and restimulated with fresh peptide-pulsed CD40-Bs and IL-7. This was repeated on days 14, 21, and 28. For restimulation of T cells, CD40-Bs were pulsed for 2 h at 37°C with peptide (10 μg/ml) and β2-microglobulin (3 μg/ml). IL-2 was first introduced into the cultures at days 18–20 (10 IU/ml). To further expand T cells, IL-2 was added at 100 IU/ml from day 24 on every third day until cytotoxicity was assessed at day 35.

Cellular cytotoxicity assay. Expanded autologous $CD8^+$ T cell lines were analyzed in a standard 4-h ^{51}Cr-release assay or by JAM-test (37, 41) for their ability to kill various target cells. As target cells, melanoma cells or CD40-Bs, alone or peptide-pulsed were harvested from culture using standard procedures for adherent respectively nonadherent cells, washed twice by centrifugation in PBS and resuspended in RPMI-5. Targets were labeled with either ^{51}Cr or ^{3}H-Tdr as previously described and 2×10^4 labeled cells were plated with various concentrations of effector cells. Percent cytotoxicity is calculated as the [(cpm-spontaneous release)/(total cpm-spontaneous release)] ×100% for chromium release assays and [(spontaneous release-cpm)/spontaneous release] × 100% for the JAM test (37, 41).

Results

Large numbers of CD40-Bs can be generated that induce highly significant peak T cell proliferation and IFN-γ production without IL-10 secretion. DCs and CD40-Bs generated from PB were compared with regard to cell surface phenotype, expansion, and capacity to present alloantigen. After 6 d of culture with GM-CSF and IL-4, DCs were isolated and then either continued in culture with GM-CSF and IL-4 for 9 additional days or cultured with either TNF-α or CD40L from days 6 to 8 to induce maturation (7, 27) (Fig. 1 *A*). The majority of cultured cells (> 83%) developed a DC-like morphology (7) and phenotype ($CD83^+$ $CD14^-$) after 6 d of culture (42). Cells cultured in GM-CSF and IL-4 became very large and adherent by day 12 with decreased expression of CD83, thereafter. Differentiation with either TNF-α or CD40L between days 6 and 8

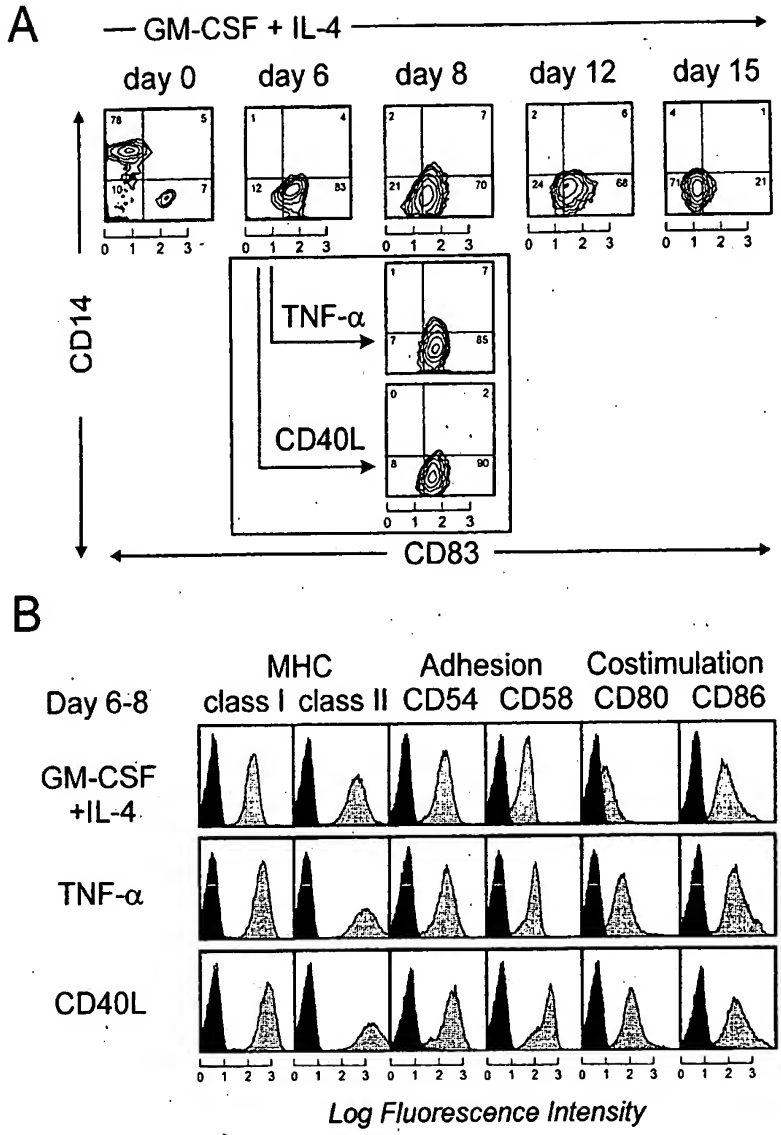


Figure 1. CD83$^+$ CD14$^-$ DCs generated from a monocyte-enriched PBMC fraction express high levels of adhesion, MHC, and costimulatory molecules. (*A*) Expression of CD14 and CD83 was assessed by two color immunofluorescence analysis before and during culture. (*B*) Expression of MHC class I and II, CD54 and CD58, CD80 and CD86 on DCs cultured for 8 d; DCs cultured with GM-CSF and IL-4 (*top*), DCs cultured with GM-CSF and IL-4 for 6 d followed by TNF-α (*middle*); or CD40L (*bottom*) for 2 d. Black shaded area indicates fluorescence of isotype matched conjugated antibodies.

did not significantly increase the expression of CD83. In contrast, the expression of MHC, adhesion, and costimulatory molecules (Fig. 1 *B*) further increased compared to already high expression on DCs cultured with GM-CSF and IL-4. However, DCs cultured for > 12 d expressed significantly lower levels of both adhesion and costimulatory molecules (data not shown).

CD40-Bs, generated by coculture of PBMC with CD40L in the presence of IL-4 and low concentrations of cyclosporin A, were > 80% CD19$^+$CD3$^-$ by day 8 and were uniformly CD19$^+$CD3$^-$ by day 12 (Fig. 2 *A*). These cells were highly activated as shown by their expression of CD23. Comparable levels of MHC, adhesion, and costimulatory molecules as observed for DCs between days 6 and 12 of culture was demonstrated for CD40-Bs at day 8 (Fig. 2 *B*) and expression of these molecules remained stable thereafter (data not shown).

DCs and CD40-Bs generated from 50 cm^3 of PB were compared for expansion in short-term cultures (Fig. 3). After 8–12 d in culture, $\sim 1 \times 10^7$ DCs could be generated and no further expansion ensued. Approximately 1×10^8 CD40-Bs could be generated by day 8 and 1×10^9 by day 14. Comparable differences in the levels of expansion were observed for DCs and CD40-Bs generated from leukophereses (Fig. 3, *bottom*).

Primary allogeneic MLRs with highly purified T cell subsets were used to compare the APC capacity of DCs and CD40-Bs. MLRs were performed for 2–8 d and peak proliferation induced by DCs was at day 7 for CD4$^+$ T cells and at day 5 for CD8$^+$ T cells (shown in Fig. 4). At early time points (2–4-d cultures, data not shown) CD40-Bs were as efficient as DCs at low APC:T ratios. However at peak proliferation (days 5–7) the most efficient alloantigen presenting cells at low APC:T cell ratios were: (*a*) DCs cultured with GM-CSF + IL-4 fol-

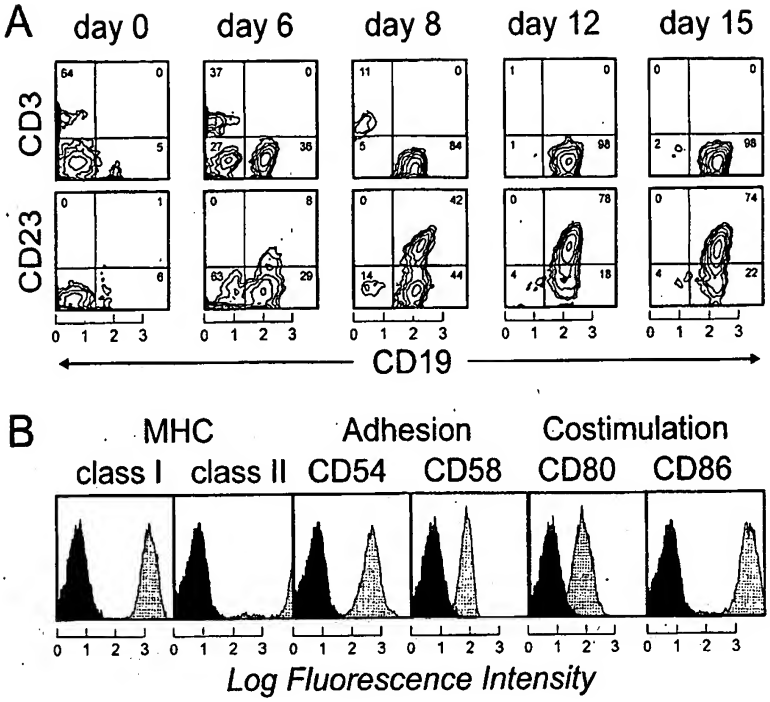
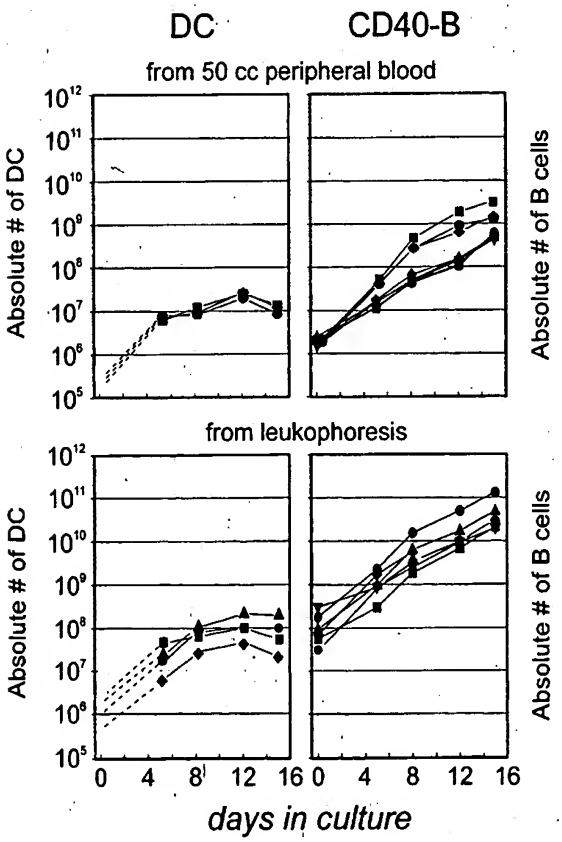


Figure 2. Stimulation of PBMC with t-CD40L and IL-4 in the presence of cyclosporin A results in outgrowth of highly pure activated B cells expressing high levels of adhesion, MHC, and costimulatory molecules. (*A*) Two color immunophenotypic analysis including CD19, CD23, and CD3 on PBMC before and during culture until day 15. (*B*) Expression of MHC class I and II, CD54, CD58, CD80, and CD86 on $CD19^+$ cells cultured for 8 d. Black shaded area indicates fluorescence of isotype matched conjugated antibodies.



lowed by CD40L activation; (*b*) DCs cultured with GM-CSF + IL-4 followed by TNF-α activation; (*c*) DCs cultured with GM-CSF + IL-4 alone; and (*d*) CD40-Bs. In all cultures (day 2–8) when $> 10^4$ DCs/well were used, T cell proliferation dramatically decreased whereas when $> 10^4$ CD40-Bs/well were used, T cell proliferation was enhanced (Fig. 4 *A*). Moreover, peak proliferation was consistently greater for CD40-Bs compared with DCs, ranging from 20 (Fig. 4) to 150% higher peak proliferation in a total of 10 experiments. Likewise, production of IFN-γ closely correlated with T cell proliferation and peak IFN-γ production was induced by CD40-Bs (Fig. 4 *B*). Of note, CD4-positive T cell subsets, both $CD45RA^+$ $CD45RO^-$ and $CD45RO^+$ $CD45RA^-$, revealed identical patterns of T cell proliferation and IFN-γ production (data not shown). Unlike DCs, CD40-Bs did not induce IL-10 production by $CD4^+$ and $CD8^+$ T cells at any stimulator/responder ratio tested. Cytokine production by T cells in cocultures with DCs or CD40-Bs was confirmed by intracellular staining for IFN-γ and IL-10 (data not shown). Taken together, although DCs are superior APC on a cell-to-cell basis, larger numbers of CD40-Bs can be

Figure 3. Expansion of DCs and CD40-Bs from PB. DCs were generated from a monocyte-enriched PBMC fraction cultured with GM-CSF and IL-4. DCs in the culture were determined by morphology and phenotype. CD40-Bs were generated from total PBMC by culture in the CD40L system. Expansion of $CD19^+$ B cells was calculated from total cell number of cells and the percentage of $CD19^+$ B cells. Expansion of DCs from either leukopheresis or PB was analyzed in four donors. CD40-B cell expansion from eight donors' PB and from six donors leukopheresis was assessed.

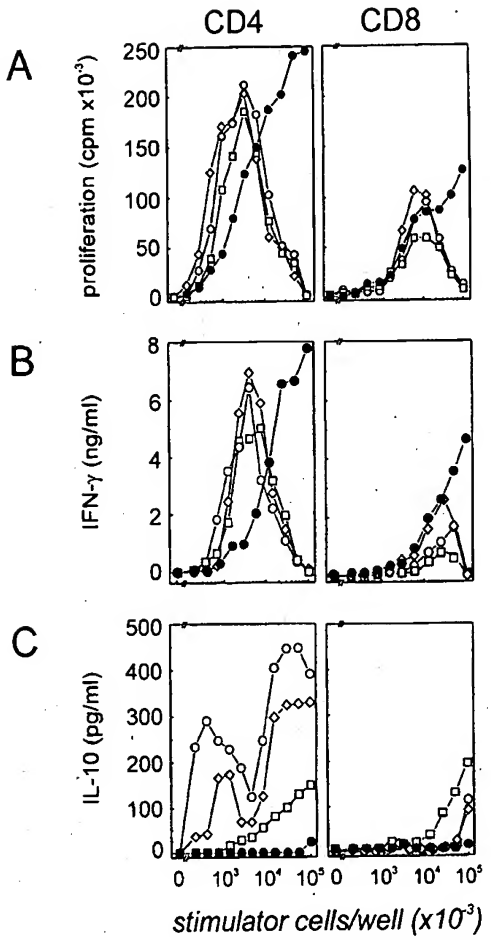


Figure 4. Response of allogeneic $CD3^+CD4^+$ T cells or $CD3^+$ $CD8^+$ T cells (10^5/well) to purified CD40-Bs or DCs. Purified T cells were cocultured with CD40-Bs (> 98% purity, *solid circles*) or PB-derived DCs cultured with GM-CSF and IL-4 (*open squares*), DCs cultured with GM-CSF and IL-4 followed by TNF-α (*open circles*), or DCs generated with GM-CSF and IL-4 followed by CD40L (*open diamonds*). [^{3}H]Thymidine incorporation was assessed for the last 16 h of 2–8 d primary MLRs. Shown here are the days of peak T cell proliferation induced by DCs (day 7 for $CD4^+$ T cells, day 5 for $CD8^+$ T cells). After 2–4 d of culture low numbers (100–10,000 cells/well) of CD40-Bs and DCs induced equivalent proliferation and IFN-γ production by $CD4^+$ T cells (data not shown). Appropriate controls (T cells, stimulator cells) were always < 2,000 cpm. Production of (*B*) IFN-γ and (*C*) IL-10 in cocultures of allogeneic T cells and either CD40-Bs or DCs was measured by ELISA in supernatants collected shortly before addition of thymidine. One representative experiment of a total of 10 experiments is shown. Phenotypic analysis of CD4 CD8 at the end of each culture demonstrated stable expression of T cell subset markers (data not shown).

generated which induce higher peak T cell proliferation and IFN-γ production without IL-10 secretion.

CD40-Bs, but not DCs, can be continuously expanded in long term culture without loss of APC function. DCs generated with GM-CSF plus IL-4 coculture could be expanded for ~ 12–15 d and cell numbers decreased dramatically thereafter with only few viable cells remaining at 4 wk (Fig. 5 *A*). Confirming the work of others (7), we observed that addition of TNF-α or CD40L to these cultures did not result in further expansion or prolonged viability of DCs (data not shown). In contrast, CD40-Bs could be continuously expanded throughout the 65 d evaluated. EBV-related proteins LMP-1 and EBNA-2 could not be detected by highly sensitive immunocytology in CD40-Bs up to day 51 of culture. When cultures were analyzed on day 65, between 5–30% of cells showed LMP-1 staining and 30–80% EBNA-2 (data not shown). However, it is critical to note that continuous CD40L and IL-4 stimulation was required and that factor-independent B cell lines were never detected. Phenotypic analysis performed weekly revealed stable expression of B cell lineage markers as well as MHC, adhesion, and costimulatory molecules (data not shown). From five unselected donors, we were able to generate between 8.5×10^{10} and 4.0×10^{11} CD40-Bs from 50 cm^3 of PB after 65 d of culture (four- to five-log fold increase). To assess APC capacity of these long-term cultured CD40-Bs, cells were harvested at days 4, 8, 15, 33, 51, and 65 of culture, cryopreserved, and used as stimulators for allogeneic $CD4^+$ T cells from three individuals. As shown in Fig. 5 *B*, unstimulated B cells were very poor APCs whereas long-term cultured CD40-Bs were highly efficient alloantigen presenting cells inducing significant T cell proliferation and IFN-γ production. In contrast to cryopreserved DCs (< 15% cell recovery, data not shown), > 75% of CD40-Bs could be consistently recovered post cryopreservation. Similar data have been obtained with CD40-Bs cultured in serum free media supplemented with autologous serum (data not shown).

Capacity of CD40-Bs to generate peptide-specific cytolytic T cells in vitro. The generation of antigen-specific cytolytic $CD8^+$ T cells for adoptive immunotherapy requires multiple stimulation with APCs in vitro. Our schema is depicted in Fig. 6. CD40-Bs were generated from PB and the remaining PBMC were cryopreserved. Once CD40-Bs had been generated, $CD8^+$ T cells were isolated and subsequently stimulated in the presence of IL-7 with peptide-pulsed CD40-Bs. From this single blood draw, continuously cultured CD40-Bs are available and can be harvested for weekly restimulations of the autologous T cells. CD40-Bs were pulsed with the immunogenic HLA-A*0201 binding 369–377 peptide YMNGTMSQV of tyrosinase, a melanoma associated tumor antigen (38). As shown in Fig. 7, *top*, T cell lines could be generated from five normal HLA-$A*0201^+$ donors that lysed either autologous or haplo-mismatched HLA-$A*0201^+$ CD40-Bs pulsed with the tyrosinase peptide from four of these donors. Most importantly, the T cell lines generated against the tyrosinase peptide-pulsed CD40-Bs demonstrated significant cytotoxicity against two HLA-$A*0201^+$ $tyrosinase^+$ melanoma cell lines. In contrast, no cytotoxicity was generated against control targets including unpulsed autologous or haplo-mismatched HLA-$A*0201^+$ (Fig. 7, *bottom*) or allogeneic $tyrosinase^+$ melanoma cells and allogeneic CD40-Bs (data not shown). To demonstrate specificity for peptide, HLA-$A*0201^+$ CD40-Bs were pulsed with an irrelevant HLA-$A*0201^+$ binding influenza A peptide. As shown, these cells were not lysed by any of the T cell lines (Fig. 7, *bottom*). These data indicate that priming with peptide-pulsed CD40-Bs induced peptide-specific cytotoxicity and that cytotoxicity was not directed against B cells, alloantigen or other processed peptides. Cold target experiments using unlabeled peptide-pulsed HLA-$A*0201^+$ CD40-Bs confirmed specificity since peptide specific cytotoxicity was abrogated (data

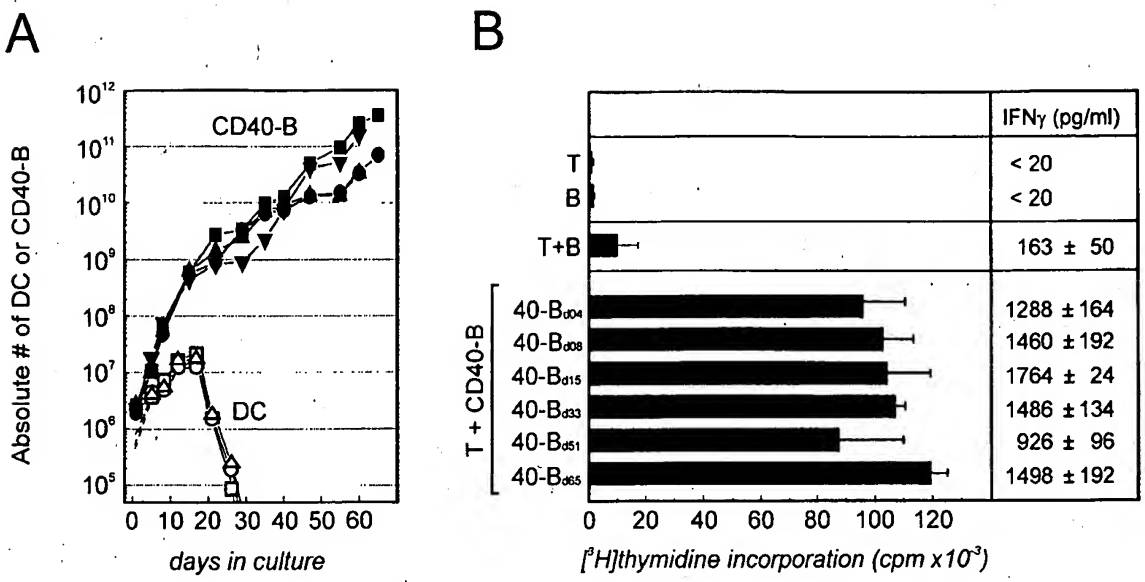


Figure 5. CD40-Bs but not DCs can be expanded in long-term culture without loss of APC function. (*A*) DCs and CD40-Bs were generated as described in Methods. Cells cultured with GM-CSF and IL-4 lost DC characteristics between day 16 and 25 of culture and the few remaining cells became very large and adherent so that cultures were stopped at day 28. Expansion of CD40-Bs was calculated as described for Fig. 2. (*B*) Induction of allogeneic T cell proliferation by unstimulated B cells or CD40-Bs precultured for 4, 8, 15, 33, 51, or 61 d. Purified $CD3^+$ $CD4^+$ T cells were cocultured with B cells or CD40-Bs in a final volume of 0.2 ml. MLRs were cultured for 5 (data not shown) or 7 d and T cell proliferation and IFN-γ production measured as described under Fig. 3. The ability of purified T cells (from other normal donors with unrelated MHC) to proliferate in response to CD40-Bs was tested in a total of three experiments.

not shown). Total number of $CD8^+$ T cells on day 35 ranged between 9×10^7 and 3×10^8.

CD40-Bs represent a cost-effective, alternative source of highly efficient antigen presenting cells. We compared the cost of preparing either DCs or CD40-Bs for repetitive ex vivo activation of antigen-specific T cells for adoptive immunotherapy (Table I). Generation of between 1×10^8 to 1×10^9 DCs ranges from two to seven times the cost of generating 1×10^{11} CD40-Bs. Moreover, generation of 1×10^8 DCs requires unacceptable phlebotomy (100 cm^3 per week for 10 wk) whereas 10^{11} CD40-Bs can be generated from a single 50-cm^3 blood draw.

Discussion

Multiple clinical trials have demonstrated the efficacy of adoptively transferred matched allogeneic antigen-specific T cells for both treatment and prophylaxis (reviewed in references 1, 2). Prophylactic transfer of cytomegalovirus specific allogeneic T cells has been successful in preventing reactivation of cytomegalovirus in immunocompromised patients after allogeneic transplant (43, 44). Treatment of EBV-induced lymphoproliferation in immunosuppressed patients with allogeneic EBV-specific T cells has resulted in clinical complete remissions (45–49) and, importantly, prophylactic trials are already

Table I. Cost Analysis of DC from Different Sources and CD40-B to Obtain APC for Ex Vivo Expansion of Autologous T Cells for Adoptive Immunotherapy

	Source*	Cell numbers‡	Preparation§	Culture‖	Estimated cost¶
					$
DC from PB	10× 100 cm^3 PB	2×10^8	10× monocyte enrichment	GM-CSF + IL-4±CD40L±TNF-α	6000
DC from LP	5× leukophereses	5×10^8	5× monocyte enrichment	GM-CSF + IL-4±CD40L±TNF-α	12000
DC from $CD34^+$	10× G-CSF 2-4 leukophereses	1×10^9	2× $CD34^+$ column cryopreservation	SCF + flt-2/flk-3L + GM-CSF + TNF-α	20000
CD40-B	1× 50 cm^3 PB	1×10^{11}		IL-4 + CD40L	3000

*Estimated amount of PB or leukopheresis (*LP*) necessary to obtain sufficient numbers of APCs to stimulate T cells ex vivo. ‡Total cell number generated from each source. §Preparation of all cells include Ficoll density centrifugation before further enrichment procedures according to previously published procedures for DCs (7, 27–33) and CD40-Bs. ‖Culture conditions according to previously published procedures to obtain highly enriched DCs (7, 27–33) or CD40-Bs (19, 37). ¶Cost analysis includes clinical visits and procedures, material, medium, cytokines, and labor.

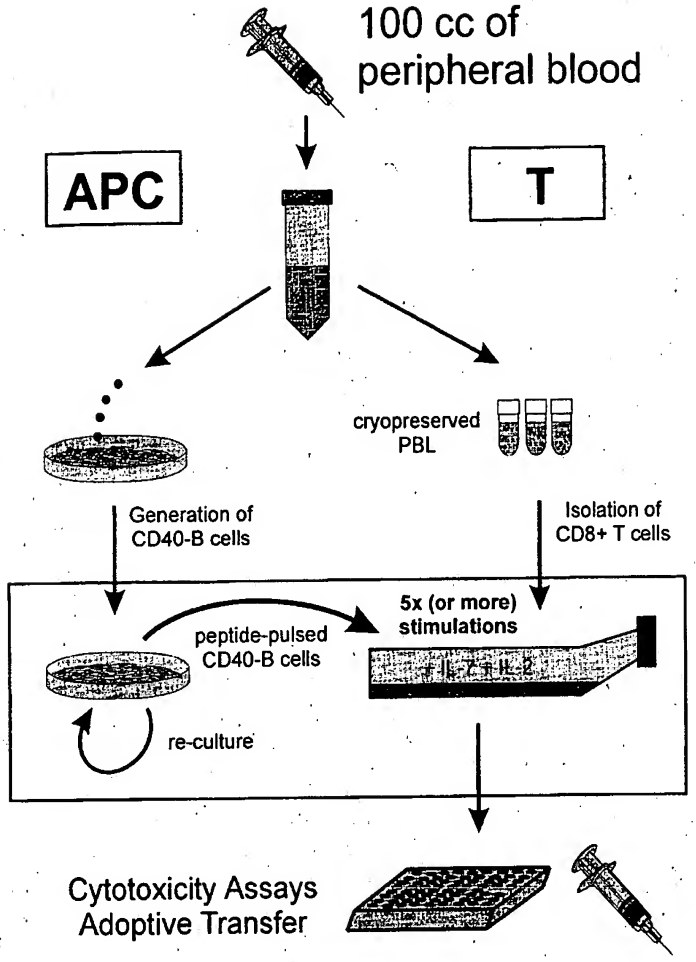


Figure 6. Induction of peptide-specific T cells by repetitive stimulation with peptide-pulsed CD40-Bs in vitro. From one single blood draw (100 cm^3) CD40-Bs as well as T cells are obtained. Half of the PBMC are used to generate CD40-Bs, the remaining cells cryopreserved for isolation of T cells. Once CD40-Bs are generated after 8–10 d, $CD8^+$ T cells can be isolated from the cryopreserved PBMC and stimulated repeatedly with peptide-pulsed CD40-Bs in the presence of IL-7. IL-2 is first added at day 18 of coculture and from day 24 on every third day thereafter.

encouraging. Finally, adoptive transfer of HLA-matched PBMC results in many complete remissions in patients with overtly relapsed chronic myelogeneous leukemia (50–53), myeloma (54, 55), and chronic lymphocytic leukemia. Therefore, the capacity to generate sufficient numbers of autologous antigen-specific T cells for adoptive immunotherapy may also provide an important therapeutic approach to treat patients with viral and fungal infections as well as with cancer.

The objective of the present study was to identify a simple, highly efficient, cost-effective source of autologous APCs with which to generate autologous antigen-specific T cells ex vivo for adoptive immunotherapy. From a single 50-cm^3 phlebotomy, very large numbers of autologous CD40-Bs can be generated. CD40-Bs are readily available for repetitive autologous T cell stimulations since they do not lose APC capacity either during long-term culture or cryopreservation and can be continuously expanded. This technology allowed us to multiply restimulate antigen-specific T cells to tyrosinase using this continuously available source of functional APC. In contrast, although DCs are highly efficient at APC function, our inability to continuously expand and/or cryopreserve mature DCs severely limited their functional utility in this setting. CD40-Bs appear to have additional unique characteristics including the induction of extremely high peak T cell proliferation, the induction of INF-γ production without IL-10 production, their simplicity of preparation, and cost. CD40-Bs might replace Epstein Barr virus–transformed lymphoblastoid cells lines (EBV-LCL) as APCs for repetitive T cell stimulation (1, 2). To our best calculation, from 50 cm^3 of PB larger numbers (10^{10}–10^{11} in 50 d) of CD40-Bs can be generated more consistently and rapidly than EBV-LCL. Moreover, if CD40-Bs cultured for < 50 d are used as APCs, they do not induce EBV-directed T cell responses during ex vivo culture. It remains to be determined, if the detection of EBV-related proteins during late cultures (day 65) predict for the outgrowth of EBV cell lines thereafter and if this might limit the culture system. Current experiments are aimed to answer these important questions.

Recent reports have demonstrated that the transfection of genes into DCs is superior to peptide pulsing in generating peptide-specific T cells (56, 57). Preliminary results in our laboratory suggest that CD40-Bs can also be efficiently transfected without changing their functional, phenotypic and/or growth characteristics. We have been able to transduce CD40-Bs with a marker gene or the human GM-CSF gene. Current studies are aimed to determine whether transfection of genes encoding for tumor antigens will lead to efficient peptide pre-

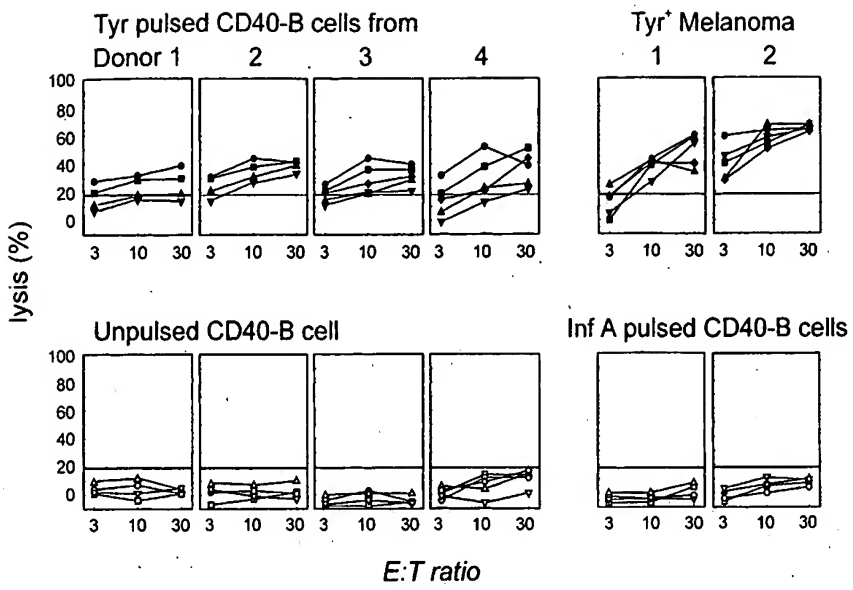


Figure 7. Induction of cytotoxic T cells after stimulation with peptide-pulsed CD40-Bs. HLA-A*0201$^+$ CD8$^+$ T cells from five normal donors were multiply stimulated with autologous CD40-Bs and subsequently analyzed for their cytotoxicity. Targets were autologous or HLA-A*0201$^+$ haplo-mismatched CD40-Bs from four donors pulsed with the specific tyrosinase peptide (*top left panel*), HLA-A*0201$^+$ melanoma cell lines positive for the tyrosinase gene (*top right panel*), unpulsed autologous or HLA-A*0201$^+$ haplo-mismatched CD40-Bs from four donors (*bottom left panel*), or autologous of HLA-A*0201$^+$ haplo-mismatched CD40-Bs from two donors pulsed with an irrelevant peptide (*Influenza A peptide*) binding to HLA-A*0201 (*bottom right panel*). Cytotoxicity was measured by JAM-test (shown here) or ^{51}Cr release with similar results.

sentation by CD40-Bs. Taken together, we conclude that CD40-Bs are an alternative highly efficient source of APCs with which to generate autologous T cells for adoptive immunotherapy.

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References

1. Riddell, S.R., and P.D. Greenberg. 1995. Principles for adoptive T cell therapy of human viral diseases. *Annu. Rev. Immunol.* 13:545–586.

2. Riddell, S.R., M.J. Gilbert, and P.D. Greenberg. 1993. CD8+ cytotoxic T cell therapy of cytomegalovirus and HIV infection. *Curr. Opin. Immunol.* 5: 484–491.

3. McKeever, D.J., and W.I. Morrison. 1994. Immunity to a parasite that transforms T lymphocytes. *Curr. Opin. Immunol.* 6:564–567.

4. Boon, T., J.C. Cerottini, B. Van den Eynde, P. van der Bruggen, and A. Van Pel. 1994. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.* 12:337–365.

5. Celluzzi, C.M., J.I. Mayordomo, W.J. Storkus, M.T. Lotze, and L.D. Falo, Jr. 1996. Peptide-pulsed dendritic cells induce antigen-specific cytotoxic T lymphocyte–mediated protective tumor immunity. *J. Exp. Med.* 183:283–287.

6. Paglia, P., C. Chiodoni, M. Rodolfo, and M.P. Colombo. 1996. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J. Exp. Med.* 183:317–322.

7. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* 179:1109–1118.

8. Steinman, R.M., M.D. Witmer-Pack, and K. Inaba. 1993. Dendritic cells: antigen presentation, accessory function and clinical relevance. *Adv. Exp. Med. Biol.* 329:1–9.

9. Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells as antigen presenting cells in vivo. *Int. Rev. Immunol.* 6:197–206.

10. Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J. Exp. Med.* 172:631–640.

11. Metlay, J.P., E. Pure, and R.M. Steinman. 1989. Control of the immune response at the level of antigen-presenting cells: a comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* 47:45–116.

12. Romani, N., S. Koide, M. Crowley, M. Witmer-Pack, A.M. Livingstone, C.G. Fathman, K. Inaba, and R.M. Steinman. 1989. Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. *J. Exp. Med.* 169:1169–1178.

13. Rock, K.L., L. Rothstein, S. Gamble, and C. Fleischacker. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J. Immunol.* 150:438–446.

14. Ke, Y., and J.A. Kapp. 1996. Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells B receptor-mediated uptake. *J. Exp. Med.* 184:1179–1184.

15. Vidard, L., M. Kovacsovics-Bankowski, S.K. Kraeft, L.B. Chen, B. Benacerraf, and K.L. Rock. 1996. Analysis of MHC class II presentation of particulate antigens of B lymphocytes. *J. Immunol.* 156:2809–2818.

16. Constant, S., N. Schweitzer, J. West, P. Ranney, and K. Bottomly. 1995. B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo. *J. Immunol.* 155:3734–3741.

17. Liu, Y.-J., C. Barthelemy, O. de Boutellier, C. Arpin, I. Durand, and J. Banchereau. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* 2:239–248.

18. Mitchell, R.N., K.A. Barnes, S.A. Grupp, M. Sanchez, Z. Misulovin, M.C. Nussenzweig, and A.K. Abbass. 1995. Intracellular targeting of antigens internalized by membrane immunoglobulin in B lymphocytes. *J. Exp. Med.* 181: 1705–1714.

19. Schultze, J.L., A.A. Cardoso, G.J. Freeman, M.J. Seamon, J. Daley, G.S. Pinkus, J.G. Gribben, and L.M. Nadler. 1995. Follicular lymphomas can be induced to present alloantigen efficiently: a conceptual model to improve their tumor immunogenicity. *Proc. Natl. Acad. Sci. USA.* 92:8200–8204.

20. Liu, K.J., V.S. Parikh, P.W. Tucker, and B.S. Kim. 1994. Surface immunoglobulins mediate efficient transport of antigen to lysosomal compartments resulting in enhanced specific antigen presentation by B cells. *Eur. J. Immunol.* 24:2755–2760.

21. Topalian, S.L., L. Rivoltini, M. Mancini, J. Ng, R.J. Hartzman, and S.A. Rosenberg. 1994. Melanoma-specific CD4+ T lymphocytes recognize human melanoma antigens processed and presented by Epstein-Barr virus-transformed B cells. *Int. J. Cancer.* 58:69–79.

22. Gollob, K.J., L. Nagelkerken, and R.L. Coffman. 1993. Endogenous retroviral superantigen presentation by B cells induces the development of type 1 CD4+ T helper lymphocytes. *Eur. J. Immunol.* 23:2565–2571.

23. Cella, M., F. Sallusto, and A. Lanzavecchia. 1997. Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* 9:10–16.

24. Hsu, F.J., C. Benike, F. Fagnoni, T.M. Liles, D. Czerwinski, B. Taidi, E.G. Engleman, and R. Levy. 1996. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat. Med.* 2:52–58.

25. Flamand, V., T. Sornasse, K. Thielemans, C. Demanet, M. Bakkus, H. Bazin, F. Tielemans, O. Leo, J. Urbain, and M. Moser. 1994. Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. *Eur. J. Immunol.* 24:605–610.

26. Girolomoni, G., and P. Ricciardicastagnoli. 1997. Dendritic cells hold promise for immunotherapy. *Immunol. Today.* 18:102–104.

27. Romani, N., S. Gruner, D. Brang, E. Kampgen, A. Lenz, B. Trockenbacher, G. Konwalinka, P.O. Fritsch, R.M. Steinman, and G. Schuler. 1994. Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* 180:83–93.

28. Romani, N., D. Reider, M. Heuer, S. Ebner, E. Kämpgen, B. Eibl, D. Niederwasser, and G. Schuler. 1996. Generation of mature dendritic cells from human peripheral blood. An improved method with special regard to clinical applicability. *J. Immunol. Methods.* 196:137–151.

29. Bender, A., M. Sapp, G. Schuler, R.M. Steinman, and N. Bhardwaj. 1996. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods.* 196:121–135.

30. Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Banchereau. 1992. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature.* 360:258–261.

31. Young, J.W., P. Szabolcs, and M.A. Moore. 1995. Identification of dendritic cell colony-forming units among normal human $CD34^+$ bone marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. *J. Exp. Med.* 182:1111–1119.

32. Szabolcs, P., M.A. Moore, and J.W. Young. 1995. Expansion of immunostimulatory dendritic cells among the myeloid progeny of human CD34+ bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor, and TNF-alpha. *J. Immunol.* 154:5851–5861.

33. Siena, S., M. Di Nicola, M. Bregni, R. Mortarini, A. Anichini, L. Lombardi, F. Ravagnani, G. Parmiani, and A.M. Gianni. 1995. Massive ex vivo generation of functional dendritic cells from mobilized CD34+ blood progenitors for anticancer therapy. *Exp. Hematol.* 23:1463–1471.

34. Banchereau, J., and F. Rousset. 1991. Growing human B lymphocytes in the CD40 system. *Nature.* 353:678–679.

35. Banchereau, J., P. de Paoli, A. Valle, E. Garcia, and F. Rousset. 1991. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science.* 251:70–72.

36. Banchereau, J., F. Bazan, D. Blanchard, F. Briere, J.P. Galizzi, C. Vankooten, Y.J. Liu, F. Rousset, and S. Saeland. 1994. The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12:881–922.

37. Schultze, J.L., M.J. Seamon, S. Michalak, J.G. Gribben, and L.M. Nadler. 1997. Autologous tumor infiltrating T cells cytotoxic for follicular lymphoma cells can be expanded in vitro. *Blood.* 89:3806–3816.

38. Visseren, M.J., A. van Elsas, E.I. van der Voort, M.E. Ressing, W.M. Kast, P.I. Schrier, and C.J. Melief. 1995. CTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. *J. Immunol.* 154:3991–3998.

39. Mellado, B., D. Colomer, T. Castel, M. Munoz, E. Carballo, M. Galan, J.M. Mascaro, J.L. Vives-Corrons, J.J. Grau, and J. Estape. 1996. Detection of circulating neoplastic cells by reverse-transcriptase polymerase chain reaction in malignant melanoma: association with clinical stage and prognosis. *J. Clin. Oncol.* 14:2091–2097.

40. Pinkus, G.S., M. Lones, I.P. Shintaku, and J.W. Said. 1994. Immunohistochemical detection of Epstein-Barr virus-encoded latent membrane protein in Reed-Sternberg cells and variants of Hodgkin's disease. *Mod. Pathol.* 7:454–461.

41. Matzinger, P. 1991. The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Methods.* 145:185–192.

42. Szabolcs, P., D. Avigan, S. Gezelter, D.H. Ciocon, M.A.S. Moore, R.M. Steinman, and J.W. Young. 1996. Dendritic cells and macrophages can mature independently from human bone marrow-derived, post-colony-forming unit intermediate. *Blood.* 87:4520–4530.

43. Riddell, S.R., K.S. Watanabe, J.M. Goodrich, C.R. Li, M.E. Agha, and P.D. Greenberg. 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science.* 257:238–241.

44. Walter, E.A., P.D. Greenberg, M.J. Gilbert, R.J. Finch, K.S. Watanabe, E.D. Thomas, and S.R. Riddell. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N. Engl. J. Med.* 333:1038–1044.

45. Heslop, H.E., C.Y. Ng, C. Li, C.A. Smith, S.K. Loftin, R.A. Krance, M.K. Brenner, and C.M. Rooney. 1996. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat. Med.* 2:551–555.

46. Lucas, K.G., T.N. Small, G. Heller, B. Dupont, and R.J. O'Reilly. 1996. The development of cellular immunity to Epstein-Barr virus after allogeneic bone marrow transplantation. *Blood.* 87:2594–2603.

47. Rooney, C.M., C.A. Smith, C.Y. Ng, S. Loftin, C. Li, R.A. Krance, M.K. Brenner, and H.E. Heslop. 1995. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet.* 345:9–13.

48. Papadopoulos, E.B., M. Ladanyi, D. Emanuel, S. Mackinnon, F. Boulad, M.H. Carabasi, H. Castro-Malaspina, B.H. Childs, A.P. Gillio, T.N. Small, et al. 1994. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N. Engl. J. Med.* 330:1185–1191.

49. Porter, D.L., G.J. Orloff, and J.H. Antin. 1994. Donor mononuclear cell infusions as therapy for B-cell lymphoproliferative disorder following allogeneic bone marrow transplant. *Transplant. Sci.* 4:12–14.

50. Giralt, S.A., and H.J. Kolb. 1996. Donor lymphocyte infusions. *Curr. Opin. Oncol.* 8:96–102.

51. Lewalle, P., N. Hensel, A. Guimaraes, D. Couriel, Y. Jiang, Z.D. Mavroudis, and A.J. Barrett. 1996. Helper and cytotoxic lymphocyte responses to chronic myeloid leukaemia: implications for adoptive immunotherapy with T cells. *Br. J. Haematol.* 92:587–594.

52. Kolb, H.J., A. Schattenberg, J.M. Goldman, B. Hertenstein, N. Jacobsen, W. Arcese, P. Ljungman, A. Ferrant, L. Verdonck, D. Niederwieser, et al. 1995. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European group for blood and marrow transplantation working party chronic leukemia. *Blood.* 86:2041–2050.

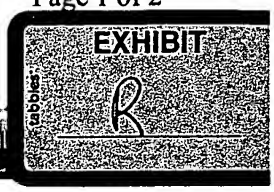
53. Porter, D.L., M.S. Roth, C. McGarigle, J.L. Ferrara, and J.H. Antin. 1994. Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia. *N. Engl. J. Med.* 330:100–106.

54. Bertz, H., J.A. Berger, R. Kunzmann, R. Mertelsmann, and J. Finke. 1997. Adoptive immunotherapy for relapsed multiple myeloma after allogeneic bone marrow transplantation (BMT): evidence for a graft-versus-myeloma effect. *Leukemia (Baltimore).* 11:281–283.

55. Verdonck, L.F., H.M. Lokhorst, A.W. Dekker, H.K. Nieuwenhuis, and E.J. Petersen. 1996. Graft-versus-myeloma effect in two cases. *Lancet.* 347:800–801.

56. Brossart, P., A.W. Goldrath, E.A. Butz, S. Martin, and M.J. Bevan. 1997. Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. *J. Immunol.* 158:3270–3276.

57. Yee, C., M.J. Gilbert, S.R. Riddell, V.G. Brichard, A. Fefer, J.A. Thompson, T. Boon, and P.D. Greenberg. 1996. Isolation of tyrosinase-specific CD8+ and CD4+ T cell clones from the peripheral blood of melanoma patients following in vitro stimulation with recombinant virus. *J. Immunol.* 157:4079–4086.



1: Thyroid 1995 Aug;5(4):259-64

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Establishment and characterization of an antihuman thyrotropin (TSH) receptor-specific CD4+ T cell line from a patient with Graves' disease: evidence for multiple T cell epitopes on the TSH receptor including the transmembrane domain.

Akamizu T, Ueda Y, Hua L, Okuda J, Mori T.

Department of Laboratory Medicine, Kyoto University School of Medicine, Japan.

From the peripheral lymphocytes of a patient with Graves' disease, we established a T cell line using its reaction to a pool of 49 synthetic peptides corresponding to the entire human thyrotropin receptor (TSHR) sequence. This T cell line showed a specific response to the pool of peptides in a microproliferation assay (stimulation index: 4.8). Flow cytometry analysis revealed that the cell surface markers were CD4+ CD8-, T cell receptor (TcR) alpha beta+, and Tcr gamma delta-. To investigate T cell epitopes on TSHR, the T cell line reacted well against three groups: the N-terminal (amino acids 31-169) and C-terminal (338-420) regions of the extracellular domain and the N-terminal half (441-661) of the transmembrane domain of the receptor. This suggests a multiplicity of T cell epitopes on the TSHR, and was further supported by analysis of TcR gene expression in the cell line that showed the expression of 5 V alpha genes; V alpha-1, 2, 10, 20, and w25. In conclusion, the results of the present study indicated multiple T cell epitopes on the TSHR molecule including the transmembrane domain.

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Autoreactive T-cells in Goodpasture's syndrome recognize the N-terminal NC1 domain on alpha 3 type IV collagen.

Merkel F, Kalluri R, Marx M, Enders U, Stevanovic S, Giegerich G, Neilson EG, Rammensee HG, Hudson BG, Weber M.

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Goodpasture's syndrome is mediated by immunopathogenic autoantibodies to the alpha 3 NC1 domain of type IV collagen. It is not known whether collaborating T-cells participate in this autoreactive response. Here we describe the first T-cell clone isolated from a Goodpasture patient autoreactive to alpha 3 type IV collagen of glomerular basement membrane. To investigate cellular autoreactivity, T-cells from Goodpasture patients or controls were isolated and stimulated by purified native or recombinant type IV collagen proteins and synthetic oligopeptides. Cell surface markers, the T-cell receptor repertoire, and MHC-restriction were analyzed. T-cell clones specific for the alpha 3 (IV) NC1 domain were established in two Goodpasture patients, but not in controls. One of the three CD8+ T-cell clones was characterized further. It was MHC class I restricted (HLA-A11) and expressed the T-cell receptor V beta 5.1. chain. This clone specifically recognized a motif at the N-terminal area of the alpha 3 (IV) NC1 domain (AA 51 to 59: GSPATWTTR). We conclude that autoreactive T-cells exists in Goodpasture patients and may play a crucial role in the inflammatory process. T-cell clones are autoreactive to the alpha 3 (IV) NC1 domain. At least for one of the clones, the T-cell epitope is different from the putative antibody-binding site.

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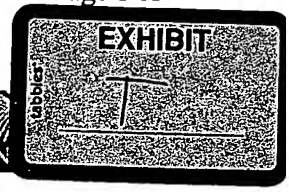

A review of T-cell receptors in multiple sclerosis: clonal expansion and persistence of human T-cells specific for an immunodominant myelin basic protein peptide.

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Understanding the immune response to myelin antigens in regard to the peptide/MHC/TCR complex is important in defining pathogenesis of demyelinating autoimmune diseases and in developing antigen-specific therapies. We previously reported that individual multiple sclerosis patients may use certain dominant TCR V beta chains to recognize immunodominant MBP peptides. In examining the TCR beta chain usage, we observed repeated TCR VDJ sequences among different T-cell lines isolated from the same patient. This suggested that a few expanded T-cell clones may dominate the immune response to immunodominant MBP peptides. Here, we report experiments where TCR rearrangements were used as a probe for the clonal origin of MBP specific T-cells cultured from blood lymphocytes of MS patients and normal subjects. In two patients with the DR2 haplotype that were analyzed in detail, the T-cell response to MBP was focused on the MBP (84-102) peptide and in vivo expanded population(s) dominated the response to the MBP (84-102) peptide. Two MBP (84-102) specific T-cell clones from a normal subject with the DR2 haplotype were also found to have identical TCR sequences. Clonality was proven by demonstrating that independent clones had identical TCR alpha and beta chain sequences as well as identical sequences of a TCR gamma chain or of a second TCR alpha chain rearrangement. These data suggest that the response to human MBP is dominated in at least some subjects by expanded clones that may persist in vivo for relatively long periods of time.

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